ANTIPROLIFERATIVE EFFECTS OF ISOFLAVONOIDS ON PROSTATE CANCER CELLS AND ANTITUMORIGENIC EFFECTS IN XENOGRAFT NUDE MICE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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This dissertation is dedicated to my caring and supportive parents, my mother Marie Thérèse Samedi, my father Lucien Jacques Lapierre, my loving grandmother Sœurette 'Marraine' Augustin, my sister Chantal and my brother Babal.

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Epidemiological studies have suggested that the phytochemical isoflavonoids may be involved in protective effects against prostate cancer. This study investigated the anticancer properties of two biologically active isoflavonoids, genistein and its precursor biochanin A, in two human prostate cancer cell lines LNCaP (androgen-sensitive) and PC-3 (androgen-independent). LNCaP or PC-3 cells cultured in the presence of these isoflavonoids for 48 hours exhibited a dose-dependent decrease in cell viability. The level of apoptosis increased with increasing concentration of the isoflavones in LNCaP cells. In contrast, PC-3 cells were more resistant to induction of apoptosis. The isoflavonoids produced a dose-dependent inhibition of DNA synthesis in both LNCaP and PC-3 with complete inhibition at 37 and 111 μM, respectively. These concentrations were subsequently used to study mechanisms of growth inhibition. In LNCaP, genistein accumulated cells in the G2/M phase, whereas biochanin produced a G1 accumulation.

In PC-3, both isoflavonoids induced a G2/M arrest. Cyclin B protein level was markedly decreased by both phytochemicals in LNCaP, and by biochanin in PC-3. The cell cycle inhibitory protein p21 expression was induced by genistein in both cell lines, whereas biochanin decreased it by 40% in LNCaP. The effects of these plant chemicals on gene expression were investigated using cDNA microarray technology. In both cell lines, genistein and biochanin altered expression of shared and unique genes which are involved in multiple cellular functions, including DNA synthesis, transcription, translation, protein degradation, signal transduction, cell proliferation, and cell adhesion. Lastly, athymic mice were implanted with LNCaP xenografts. In mice treated with biochanin at 400 μg/day for 10 days, mean tumor volume was significantly smaller compared to controls at 3 and 6 weeks, and tumor incidence was 54% compared to 89% in control at 3 weeks. In mice with established tumors, genistein and biochanin treatment slowed the rate of growth compared to controls. In one of two experiments, biochanin significantly decreased the mitotic index in the tumors, whereas in another one genistein increased it. No changes in microvessel density were observed with biochanin treatment. These results indicate that genistein and biochanin have antiproliferative effects both in vitro and in vivo, characterized by shared and distinct mechanisms.

CHAPTER 1
INTRODUCTION

Study Objectives

Cancer of the prostate (Figure 1-1) is the second leading cause of cancer deaths in American males. In the year 2000, an estimated 180,400 new cases and 31,900 deaths were reported in the United States (Woolam, 2000). The highest rates of prostate cancer are observed in populations with Western lifestyles that include relatively high fat, meat-based, low fiber diets. In contrast, the lowest rates are typically seen in populations with Eastern lifestyles that include plant-based diets with a high content of soy products (Adlercreutz, 1990; Parkin and Muir, 1992; Rose *et al.*, 1986). The reported incidence varies from 5 to 70-fold between populations. Studies on Asian immigrants in the US indicate that this striking geographical difference in incidence rate is evidence that environmental factors are significant (Kolonel *et al.*, 1985; Kolonel *et al.*, 1988). Asian men who migrate to America tend to adopt the prostate cancer incidence of the indigenous population within one or two generations. These epidemiological data support the concept that environmental factors, including diets, may inhibit the promotion and progression of prostate cancer in Asian men. Thus it has been suggested that a diet rich in plants including soy foods, which are widely used in Asian cultures, may confer some level of protection against prostate cancer (Adlercreutz and Mazur, 1997; Barnes *et al.*, 1995; Dunn, 1975). Some of the most bioactive compounds found in soy are the isoflavonoids.

This research was undertaken to investigate the anticancer properties and modes of action of these phytochemicals, specifically genistein and biochanin A. Asian men have high urinary and plasma levels of these isoflavonoids compared to American and Western European men with low levels (Adlercreutz *et al.*, 1993). The compounds genistein and biochanin A have been reported to inhibit the proliferation of many cancer cell lines through multiple mechanisms (Bergamaschi *et al.*, 1993; Peterson and Barnes, 1991, 1993; Shao *et al.*, 1998; Spinozzi *et al.*, 1994; Yanagihara *et al.*, 1993). The hypothesis of this study is that the isoflavones genistein and biochanin exhibit their antiproliferative effects in prostate cancer cells through cell cycle arrest by modifying the expression of cell cycle regulators. The first objective was to determine the effects of the isoflavones on cell proliferation, cell cycle phase distribution and on key cell cycle regulatory proteins in two established prostate cancer cell lines LNCaP (androgen-responsive) (Horoszewicz *et al.*, 1980) and PC3 (androgen-independent) (Kaighn *et al.*, 1979). A second objective was to evaluate in vivo the chemopreventive and antiproliferative effects of these plant compounds in xenograft nude mice. Finally, a third objective was to obtain a profile of the effects of genistein and biochanin A on gene expression in the prostate cancer cell lines. Such an approach provides a global view of the net effects of these compounds in the cancer cells.

Prostate Cancer

Incidence and Mortality Rates

Prostate cancer incidence rates vary widely from one geographical location to another widely by almost 70-fold around the world (Parkin and Muir, 1992). Men living in the Far East and on the Indian subcontinent have the lowest recorded rates, whereas

men from Western Europe, Australia and North America present the highest incidence rates. After adjusting the rates to a common age standard, the incidence rate for prostate cancer was 1/100,000 men per year in Asia compared to 62 and 82/100,000 for US whites and blacks, respectively in the late 1980s. Differences in mortality rates among nations have also been noted. In Japan 4/100,000 men die of prostate cancer each year. However, the mortality rate is approximately 18/100,000 men annually, in Canada, France, Germany, the United Kingdom, and the United States (Parker *et al.*, 1998). The American Cancer Society reported, in the US only, an estimated 180,400 new cases and 31,900 deaths in 2000. Prostate cancer is the second leading cause of cancer death in American men, and the third most common cause of cancer death in the US (Woolam, 2000). The lifetime risk of being diagnosed with this cancer is 1 in 6 (Parker *et al.*, 1997).

Risk Factors

Genetics. The evidence for genetic predisposition is the presence of familial clustering, especially extended families with many cases, which suggests an inherited susceptibility (Eeles *et al.*, 1997; Gronberg *et al.*, 1997a, 1997b). The clusters described so far, however, are usually small (3-4 cases). First-degree relatives (fathers and brothers) of prostate cancer patients have been noted to have a relative risk of 3 of developing this disease (Fotsis *et al.*, 1997; Ruijter *et al.*, 1999).

The tentative hereditary prostate gene (HPC1) is linked to the long arm of chromosome 1 (1q24-25) in 30% of the families analyzed (Smith *et al.*, 1996). Another described inherited predisposition is the type of androgen receptor gene (Trapman and Brinkmann, 1996). There are short and long subtypes of the androgen receptor,

depending on how many CAG nucleotide repeats are in the receptor. Ligand-bound androgen receptor acts more strongly on the DNA target if there are fewer CAG repeats (Trapman and Brinkmann, 1996). Epidemiologic evidence indicates that the androgen receptor is long in Asian populations, medium in white populations, and short in African populations (Giovannucci *et al.*, 1997; Irvine *et al.*, 1995; Kantoff *et al.*, 1998; Platz *et al.*, 1998). The incidence of prostate cancer in these ethnic groups is inversely correlated with the length of the receptor, and it is particularly higher in African-Americans (Morton, Jr., 1994; Ross *et al.*, 1987; Wingo *et al.*, 1996). Furthermore, dihydrotestosterone has a high affinity for the androgen receptor. This ligand is produced from testosterone by the enzyme 5α-reductase type II (Vermeulen *et al.*, 1972). A TA dinucleotide repeat has been described in this in the 3'-untranslated region of the gene for this enzyme (Davis and Russell, 1993; Makridakis *et al.*, 1997, 1999; Reichardt *et al.*, 1995). African Americans tend to have longer TA alleles. Being homozygous for the longer TA alleles in 5α-reductase type II may increase the risk of prostate cancer in some cases (Kantoff *et al.*, 1997).

Diet. Ecologic studies show that per capita fat consumption is strongly correlated with prostate cancer mortality rates internationally, suggesting that dietary fat may influence the occurrence and/or progression of prostate cancer (Armstrong and Doll, 1975). The positive association between intake of dietary fat or higher-fat foods (e.g., meat and dairy foods) has been supported in case-control studies conducted in several different populations and adjusted for potentially confounding factors (Graham *et al.*, 1983; Talamini *et al.*, 1992; West *et al.*, 1991; Whittemore *et al.*, 1995). Men who consumed less fat have been shown to have lower plasma testosterone levels (Howie and

Shultz, 1985). Testosterone is the precursor of DHT, which is a growth signal for prostate cells. Also, in the case of the androgen receptor, specific fatty acids may induce changes in ligand-receptor binding or modulate receptor activity (Gann *et al.*, 1994, 1996).

There is a positive relationship between calcium and prostate cancer risk independent of higher fat content of foods containing calcium (Whittemore *et al.*, 1995). Higher circulating and tissue levels of calcium downregulate production of the steroid hormone 1,25-dihydroxyvitamin D. This steroid plays a role in control of proliferation and differentiation of cells, including prostatic epithelial cells. Decreased levels of vitamin D has been associated with increased cell proliferation. In addition, countries with greater per capita milk consumption have higher national prostate mortality rates (La Vecchia *et al.*, 1991; Rose *et al.*, 1986). The magnitude of this correlation is greater than for other foods that are high in animal fat, suggesting that dairy products have an adverse effect beyond that of fat alone (Ross *et al.*, 2000). Men consuming greater amounts of milk and other dairy products are at increased risk of prostate cancer in case-control (Rotkin, 1977; Talamini *et al.*, 1992) and prospective cohort studies (Chan *et al.*, 1998a; Le Marchand *et al.*, 1994; Snowdon *et al.*, 1984).

Molecular Progression

The epidemiological profiles of prostate cancer have been well described for many decades (Carter and Coffey, 1990). The etiology and the carcinogenesis of the prostate gland remain, nevertheless, poorly understood. It is not clear why some carcinomas remain "clinically silent" during life, whereas other tumors progress to present clinically and may lead to prostate-related death (Carter and Coffey, 1990; Chan

et al., 1998b). In autopsy studies, the prevalence of latent prostate tumors (microscopic foci of well-differentiated cancer cells) is unexpectedly high in older men, regardless of race and geographical location (Rose *et al.*, 1986). Prostate cancer is characterized by multiple molecular markers of progression at various stages (Ozen and Pathak, 2000). The development of prostate cancer is a result of a series of acquired somatic genetic changes affecting a number of genes on several chromosomes (Ozen and Pathak, 2000). The morphological and molecular changes include the progressive disruption of the basal cell layer, changes in the expression of differentiation markers of the prostatic secretory epithelial cells, nuclear and nucleolar abnormalities, increased cell proliferation, DNA content alterations, and chromosomal and allelic losses (Bostwick *et al.*, 1996; Weinberg and Weidner, 1993). Primary prostate cancer is an endocrine-related cancer and depends on the level of endogenous androgens. In fact, the cornerstone therapy is repression of androgen biosynthesis, or blockage of androgen signal tranduction pathway, which can be used effectively for the treatment of androgen-dependent prostate cancer patients (Huggins and Hodges, 1972). However, most tumors eventually show a critical relapse, which is reflected by the growth of androgen-independent cells and the progression towards hormone-refractory disease (Bubendorf *et al.*, 1999; Taplin *et al.*, 1995). This progressive gain or loss of a variety of biomarkers allows windows of opportunities for therapeutic intervention, which may halt gene alterations that are involved in the events leading to the transformation of normal prostatic epithelial cells to preneoplastic cells and malignant prostate cells.

Nutrition and Chemoprevention

The development of life-threatening prostate cancer is the culmination of a complex series of initiation and promotional events, over a period of decades and under the influence of many interacting genetic and environmental factors (Bostwick *et al.*, 1996; Ozen and Pathak, 2000; Weinberg and Weidner, 1993). The wide range in age-adjusted mortality rates for prostate cancer observed among various countries can not be attributed primarily to genetics (Zaridze and Boyle, 1987), since within ethnic groups that migrate from low to high risk areas, mortality from prostate cancer increases significantly. A rapidly growing scientific literature provides strong evidence for the hypothesis that nutrition is an important factor in modifying the risk of prostate (Clinton and Giovannucci, 1998).

Epidemiological evidence supports the idea that nutritional components may inhibit the promotion and progression of prostatic cancer in Asian men. As discussed earlier, it has been suggested that a diet rich in plants including soy products may confer some level of protection against cancer, including prostate cancer (Su *et al.*, 2000; Adlercreutz and Mazur, 1997; Barnes *et al.*, 1995). Among the components of the soy diet reported to have most of the anticancer properties are the isoflavonoids. Asian men have high urinary and plasma levels of isoflavones compared to American and European men with low levels (Adlercreutz *et al.*, 1993). It was suggested that low prostate cancer risk in Asia is due to the isoflavonoids that inhibit the growth of the malignant cells, and that these phytochemicals might be used in chemoprevention (Barnes *et al.*, 1995). Chemoprevention is the purposeful administration of natural or synthetic substances to interrupt carcinogenic processes before they result in a clinical condition (Mettlin, 1997).

Prostate cancer has been described to follow the traditional multistep nature of carcinogenesis, initiation, promotion and progression (Hennings *et al.*, 1993). Chemoprevention with isoflavonoids would seek to block the transition of a normal prostate cell to a malignant one by interfering with specific biological functions (Figure 1-2) that would confer growth advantages to transformed or DNA damaged cells (Swan and Ford, 1997).

Isoflavonoids

Source

The first recorded use of soy is contained in the Materia Medica of the Chinese Emperor Shen Nung in 2838 B.C. (Barnes, 1998). People from Southeast Asia have used soybeans as a staple for thousands of years. The proteins from soy foods comprise 20 to 60% of their daily protein intake (Lieberman, 1996). Some of the most bioactive nonnutrients of soy-based products are the isoflavonoids, which are divided into two subfamilies, isoflavones (e.g. genistein, biochanin A, daidzein) and coumestans (e.g. coumestrol) (Figure 1-3). The isoflavonoids belong to a bigger family called phytoestrogens, which are estrogenic compounds found in plants (Kurzer and Xu, 1997). These phytochemicals can be found in various plants and food items (Table 1-1). Soybeans and soy foods are the most significant sources of the isoflavones. After normalization for differences in molecular weights of the isoflavones, soy foods contain approximately 0.2 to 1.6 mg of isoflavones per gram of dry weight. Chick peas and other legumes, as well as clover, toothed medic, and bluegrass, have also been identified as isoflavone sources (Kurzer and Xu, 1997). For the coumestans, like coumestrol, the most significant sources in foods are sprouts of clover and alfafa (Franke *et al.*, 1995), with

coumestrol content of 5.6 and 0.7 mg/g dry weight, respectively. Split peas, kala chana seeds, pinto bean seeds, lima bean seeds and soybean sprouts also contain small amounts of coumestrol, 15 to 80 μg/g dry weight (Franke *et al.*, 1995).

Structure and Metabolism

The isoflavonoids are strikingly similar in chemical structure to the mammalian estrogens (Figure 1-4) (Setchell, 1998). The phenolic ring is a key structural element of most compounds that bind to the estrogen receptor. The structure of the isoflavone metabolite equol can be overlaid with the one of estradiol, with the distance between the hydroxyl group at each end of both molecules being almost identical (Setchell and Cassidy, 1999). In foods, the isoflavones are conjugated with a glucose moiety to form glycosides. Some of the major glycosides are genistin and daidzin, which are metabolized by intestinal glucosidases to their aglycone form, genistein and daidzein, respectively (Miksicek, 1995). In addition, in plants, some isoflavones such as biochanin A and formononetin are converted by demethylases to genistein and daidzein, respectively. After consumption of the isoflavones, absorption and utilization require a series of deconjugation and conjugation steps. Absorption is facilitated by hydrolysis of the sugar moiety by human gut bacterial β-glucosidases, and gastric hydrochloric acid (Kelly *et al.*, 1993; Murkies, 1998). After absorption in the small intestine, isoflavones are conjugated with glucuronic acid and sulfate by hepatic phase II enzymes (UDP-glucuronosyl transferases) (Kurzer and Xu, 1997). The conjugates are excreted through both urine and bile, as well as undergoing enterohepatic circulation. After excretion into the bile, conjugated isoflavones can be deconjugated once again by gut bacteria.

Deconjugation may promote reabsorption, further metabolism, and degradation in the lower intestine (Kurzer and Xu, 1997).

Anticancer Properties

Inhibition of cell proliferation. Uncontrolled cell proliferation is one hallmark of tumor growth, which leads to malignancy. Isoflavonoids, especially genistein, have been shown to inhibit the proliferation of many established cancer cell lines in vitro, including prostate cancer cell lines (Hempstock *et al.*, 1998; Kyle *et al.*, 1997; Onozawa *et al.*, 1998). Genistein inhibits prostate cancer cells that are androgen-responsive (LNCaP) and androgen-independent (PC-3, DU-145) (Peterson and Barnes, 1993). The inhibitory effects are thus independent of the presence of androgen receptors. Although the isoflavonoids share structural and functional activities, their effects are also independent of the presence of the estrogen receptors. Genistein inhibits proliferation similarly in estrogen receptor-positive (MCF-7, T47D) and negative (MDA-MB-231, MDA-MB-468) human breast carcinoma cell lines (Peterson and Barnes, 1991; Shao *et al.*, 1998). In addition, 37 μM of genistein and biochanin A inhibit the proliferation of many stomach cancer cell lines including HSC-45M2, HSC-41E6), esophageal cancer cell line (HEC-46R1), colon cancer cell lines (HCC-44B2, HCC-50D3) (Yanagihara *et al.*, 1993). In comparison, genistein at 111 μM inhibits proliferation of leukemic cell lines (HL-60, MO7e) (Bergamaschi *et al.*, 1993). The inhibitory effects of most of the other isoflavonoids have not been investigated.

Inhibition of tyrosine-specific protein kinases. Tyrosine kinases are necessary for the proper function of many growth factor receptors, including those for epidermal growth factor, platelet-derived growth factor, insulin and insulin-like growth factors

(Hunter and Cooper, 1985). Retroviral oncogenes, such as src, abl, yes, fes and ros, all code for tyrosine-specific protein kinases (Bishop, 1983). Tyrosine phosphorylation plays an important role in signal transduction, cell proliferation and cell transformation. It has been reported that genistein is a specific inhibitor of tyrosine kinase activity (Akiyama *et al.*, 1987; Chang and Geahlen, 1992; Levitzki and Gazit, 1995). In vitro experiments with the epidermal growth factor receptor (EGF-R) from the plasma membrane of human epidermoid carcinoma A-431 cells showed that genistein inhibited EGF-R autophosphorylation of protein tyrosine kinases, as well as phosphorylation of both natural and artificial substrates, with IC_{50} values of 2.6 to 37 μM (Ogawara *et al.*, 1989). However, it has been reported that the concentration of genistein that inhibits proliferation of prostate cancer cell lines (LNCaP and PC-3) does not inhibit tyosine kinase activity of EGF-R (Peterson and Barnes, 1993). Inhibition of protein tyrosine kinases has been proposed to explain the growth-inhibiting effects of genistein in a number of different human cancer cells, but these effects can not be explained solely through inhibition of tyrosine kinase activity. Although much focus has been on genistein, very little data are available regarding the effects of other isoflavonoids on protein tyrosine kinase activity, or in prostate cancer cell lines specifically.

Suppression of angiogenesis. Neovascularization, or angiogenesis, involves the formation of new capillaries. This process initiates the proliferation and migration of endothelial cells (Folkman, 1985). Neovascularization is normally restricted to wound healing but it is enhanced in association with tumor growth. New capillary blood vessels are necessary for a tumor to expand beyond 2 mm in size (Folkman *et al.*, 1989). Therefore, angiogenesis has a crucial role in cancer progression and malignancy

(Weidner *et al.*, 1991). Promotion of this process is probably due to the production of growth factors by the cancer cells, and fibroblast growth factors (FGF), endothelial growth factors (VEGF), or members of the FGF family (Weidner *et al.*, 1991).

Genistein has been shown to suppress angiogenesis (Fotsis *et al.*, 1993, 1995, 1997, 1998). Endothelial cells co-treated with the potent angiogenic factor basic fibroblast growth factor and genistein exhibit delayed proliferation. It has been proposed that this inhibition may occur through several ways (a) as a consequence of the competitive inhibition of ATP binding to the catalytic domain of tyrosine kinase; (b) as a consequence of the attenuation of S6 kinase, an enzyme that is also activated by basic fibroblast; or (c) through the modulation of topoisomerase I and II (Kurzer and Xu, 1997; Molteni *et al.*, 1995). Little is known about the effects of other isoflavonoids on angiogenesis.

Induction of cell cycle arrest. Progression of the cell cycle is carefully coordinated by a series of events that culminate in DNA synthesis and cell division. This cycle consists of four phases G1, S, G2/M and G0. Cells have to move through the successive phases of the cell cycle, in order to divide. A more detailed description of the cell cycle is provided in chapter 3.

Genistein arrests cell growth at various stages of the cell cycle. At 20-40 μM genistein, Jurkat T leukemia cells were arrested at the G2/M transition, whereas at 70-110 μM cells were arrested in the S-phase (Spinozzi *et al.*, 1994). Genistein also arrested K-562 leukemia cells (Kanatani *et al.*, 1993), HL-60 cells (Traganos *et al.*, 1992) and HGC-27 gastric cancer cells (Matsukawa *et al.*, 1993) at the G2/M transition. Human prostate carcinoma cells are arrested at G2/M phase with genistein (Choi *et al.*, 2000). Little, if

anything, is known about how genistein affects the key cell cycle regulating proteins, and the cell cycle pathways that may be involved. In addition, other isoflavonoids have not been investigated at all.

Induction of apoptosis. Based on morphological, biochemical, and molecular criteria, two distinct modes of cell death can be recognized: necrosis and apoptosis. Necrosis is a degenerative process resulting from injury. In contrast, activation of apoptosis is a signal transduction pathway that is highly regulated and energy dependent. Apoptosis requires participation of the cells in their demise (Arends *et al.*, 1994). The most characteristic features of apoptotic cells are chromatin condensation, extensive surface blebbing, and nuclear DNA fragmentation (Wyllie, 1992). A more detailed description of programmed cell death is given in chapter three.

Genistein has been shown to induce apoptosis in gastrointestinal and leukemic cell lines (Bergamaschi *et al.*, 1993; Yanagihara *et al.*, 1993) with the cytotoxic concentration of being 30 to 40 μM. In HL-60 cells, both genistein and tyrphostin AG82 (a synthetic tyrosine kinase inhibitor) caused apoptosis (Bergamaschi *et al.*, 1993). Genistein must have additional or different targets compared with tyrphostin since the action of genistein was partially blocked by the tyrosine phosphatase inhibitor orthovanadate, whereas that of the tyrphostin was fully blocked.

Inhibition of tumorigenesis. Soya-bean isoflavones inhibit experimental carcinogenesis in a wide variety of systems. In various models of animals exposed to carcinogenic agents, the chemopreventive effects of soy and isoflavones have been reported in 17 out of 26 (65%) studies (Adlercreutz *et al.*, 1992; Barnes, 1995). The cancers studied include those of the breast, prostate, liver, colon, stomach, bladder and

skin. Isoflavonoids inhibit the growth of tumors in chemical carcinogenesis models. Biochanin A inhibits the metabolic activation of benzo[a]pyrene (Chae *et al.*, 1991, 1992). In rat colon cancer models, genistein inhibited aberrant colonic crypt formation induced by two different carcinogens (Pereira *et al.*, 1994; Thiagarajan *et al.*, 1998). Genistein has also inhibited the incidence, multiplicity and tumor size in the DMBA-initiated, phorbol ester-promoted skin cancer model in mice (Wei *et al.*, 1995). It was found that administration of genistein only during the neonatal period lowered mammary tumor incidence, multiplicity and size, and increased latency in the DMBA-induced breast cancer model (Lamartiniere *et al.*, 1995, 1998).

Although most studies have used chemical carcinogens, a few have used xenotransplantation of human cells as the model for tumorigenesis. Xenotransplantation involves transplanting human cancerous cells into immunocompromised animals. Tumors will grow at the site of injection. Biochanin A, but not genistein, suppresses growth of SC-45M2 and HSC-41E6 stomach cancer lines in athymic nude mice (Yanagihara *et al.*, 1993). This divergent effect of these two isoflavonoids may be the result of metabolism in the animals. Most of these studies have not examined the effects of other phytoestrogens.

Alternative modes of action. There have been other mechanisms proposed to explain the effects of the isoflavonoids. Genistein has been shown to inhibit both topoisomerases I and II (Constantinou *et al.*, 1990; Hande, 1998; Markovits *et al.*, 1989; Okura *et al.*, 1988). There are a number of studies which show that isoflavones exert antioxidant effects in vitro and in vivo (Frenkel, 1992; Naim *et al.*, 1976; Pratt, 1993). In addition, a soy diet has been associated with increase in the level of sex hormone binding

globulins (SHBG) which could decrease the level of free testosterone (T) in the plasma, thus inhibiting growth of the prostate (Rubens *et al.*, 1974; Vermeulen *et al.*, 1972). Conversion of T to DHT can be prevented by isoflavones because they can inhibit 5α-reductase (Evans *et al.*, 1995), the enzyme responsible for the conversion. The isoflavonoids have weak estrogenic activity with relative potencies (compared to estradiol, to which an arbitrary value of 100 was given, and determined by human cell culture bioassays) reported to be the following, coumestrol 0.202, genistein 0.084, daidzein 0.013 (Hughes, 1996; Lieberman, 1996). The isoflavonoids have multiple biological functions, with no receptor being defined to date.

Prostate Cancer Cell Lines

LNCaP Cell Line

This human cancer cell line has an epithelial morphology, and it was established from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma (Horoszewicz *et al.*, 1980). LNCaP cells grow readily in vitro with a doubling time of 32 hours, form colonies in semisolid media, and develop into tumors when implanted in nude mice with Matrigel® (Lim *et al.*, 1993; Horoszewicz *et al.*, 1983). The malignant properties of these cells are maintained, but the line expresses a functional p53 tumor suppressor protein (Carroll *et al.*, 1993; Rokhlin *et al.*, 2000).

The LNCaP cell line is androgen-sensitive (Schuurmans *et al.*, 1988b, 1988a), which is a feature of an early stage of prostate cancer. Furthermore, this cell line contains an abnormal androgen receptor with broad ligand binding specificity (Veldscholte *et al.*,

1990a). Progestagens, estradiol and several antiandrogens compete with androgens for binding to the mutated androgen receptor, and these ligands have growth stimulatory effects in the LNCaP cells (Veldscholte *et al.*, 1990b, 1992a; Olea *et al.*, 1990). This androgen receptor contains a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand binding domain (Veldscholte *et al.*, 1992b).

PC-3 Cell Line

This human cancer cell line is also of epithelial origin, and it was established from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old Caucasian male (Kaighn *et al.*, 1979). They have a doubling time of 32 hours. PC-3 cells show anchorage-independent growth in both monolayers and in soft agar suspension, and they can produce subcutaneous tumors in nude mice. Unlike LNCaP cells, PC-3 cells show a very reduced dependence upon serum for growth. The latter do not respond to androgen deprivation, which is a key feature of advanced prostatic cancer cells (Kaighn *et al.*, 1979). Furthermore, PC-3 does not express a functional p53. This line has a single copy of the p53 gene, which has a base pair deletion at codon 138 that generated a frame shift and a new in-frame stop codon at position 169. PC-3 cells are, therefore, more resistant to exogenous negative insults to proliferation (Carroll *et al.*, 1993).

Study Outline

The first goal of the study is to evaluate the antiproliferative effects of the isoflavones in the two prostate cancer cell lines LNCaP (androgen-responsive) and PC3 (androgen-independent). The second goal is to assess the antitumorigenic effects of the isoflavones in xenograft nude mice. Lastly, the effects of the isoflavones on gene

expression, in the prostate cancer the two cell lines, are to be investigated using cDNA microarray analysis.

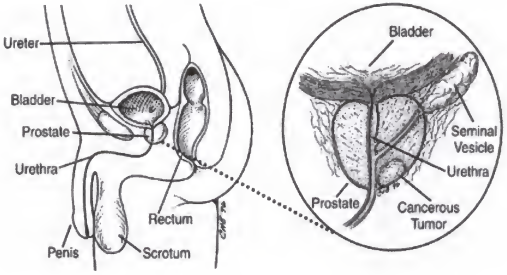


FIGURE 1-1. The prostate. This male organ is a walnut-sized gland located in front of the rectum, at the outlet of the bladder. Male hormones stimulate the prostate gland to develop in the fetus (American Cancer Society, www.cancer.org).

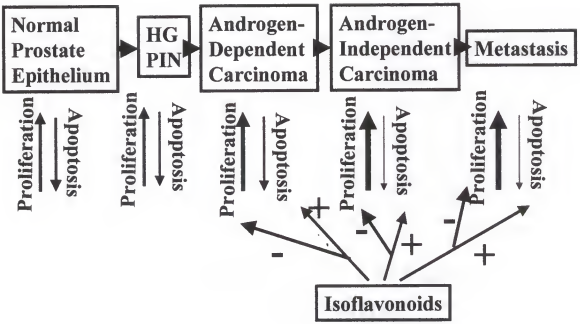


FIGURE 1-2. Concept of chemoprevention with isoflavonoids. Exposure of prostate cells to chemopreventive agents, like the isoflavonoids, would block the successive transformation from normal to malignant cancer cells (adapted from Tang and Porter, 1997).

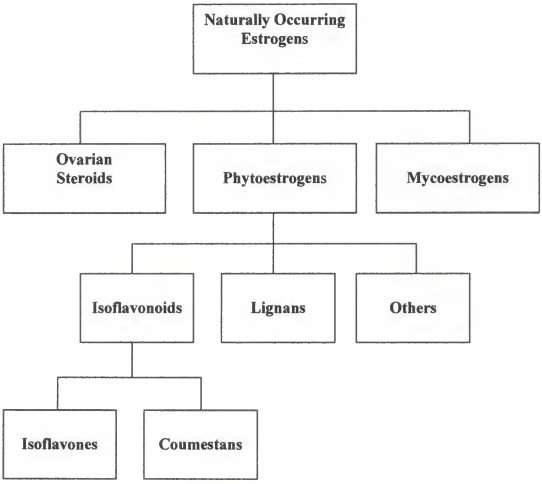
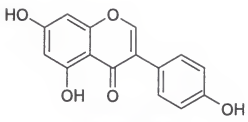
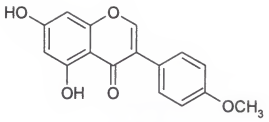


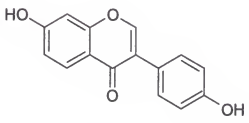
FIGURE 1-3. Classification of naturally occurring dietary estrogens. Genistein, biochanin A and daidzein are isoflavones. Coumestrol is a coumestan (adapted from Murkies et al., 1998).



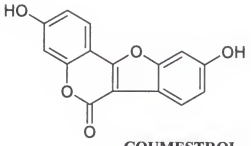
GENISTEIN



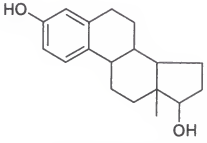
BIOCHANIN A



DAIDZEIN



COUMESTROL



ESTRADIOL

FIGURE 1-4. Structures of isoflavonoids. Genistein (4',5,7-trihydroxyisoflavone), biochanin A (5,7-dihydroxy-4'-methoxyisoflavone), daidzein (4',7-dihydroxyisoflavone), coumestrol (7,12-dihyroxycoumestan) and estradiol.

Table 1-1. Food content of isoflavonoids. The levels in food items are analyzed by HPLC (adapted from Kurzer and Xu, 1997). Values are represented as mg/kg of food material, and are means of repeated analyses (2 to 6 times) from dry or freezedried item with relative standard deviations between 3 and 11%. Food items with different numbers derived from different sources.

Food Item	Genistein	Biochanin A	Daidzein	Coumestrol
Soy bean seeds 1 (dry)	1002.7	nd	1001.3	nd
Soy bean seeds 2 (dry)	1082.0	nd	700.6	nd
Soy bean seeds 3 (dry)	1382.4	nd	1006.5	nd
Soy bean seeds 3 (roasted)	1105.5	nd	848.1	nd
Soy bean seeds 4 (raw, fd)	257.0	nd	252.0	nd
Soy bean seeds 5 (boiled, fd)	227.4	nd	224.7	nd
Soy bean seeds 6 (frozen, fd)	825.7	nd	738.5	nd
Soy bean hulls 6 (frozen, fd)	74.1	nd	nd	nd
Soy flour	1122.6	nd	654.7	nd
Tofu (fd)	1232.7	nd	840.2	nd
Black soybean seeds 1 (dry)	612.2	nd	698.5	nd
Black soybean seeds 2 (boiled, fd)	796.4	nd	774.4	nd
Black bean seeds	nd	nd	nd	nd
Green beans 1 (raw, fd)	nd	trace	nd	nd
Green beans 2 (boiled, fd)	nd	trace	nd	nd
Large lima bean seeds (dry, raw)	nd	nd	nd	14.8
Large lima bean seeds (boiled, fd)	nd	nd	nd	nd
Red bean seeds (dry)	3.1	nd	nd	trace
Garbonzo bean seeds (dry)	nd	15.2	nd	nd
Kidney bean seeds (cooked, fd)	nd	13.2	nd	nd
Pinto bean seeds (dry)	nd	5.6	nd	36.1
White navy bean seeds (dry)	nd	trace	nd	nd
Small lima bean seeds (dry)	nd	3.7	nd	nd
Great northern bean seeds (dry)	nd	6.0	nd	nd
Broad bean seeds (fried)	12.9	nd	nd	nd
Pink bean seeds (dry)	nd	nd	nd	nd
Black eyed bean seeds (dry)	nd	17.3	nd	nd
Small white bean seeds (dry)	7.4	nd	nd	nd
Yellow split peas (dry)	nd	8.6	nd	nd
Green split peas (dry)	nd	nd	72.6	nd
Round split peas (dry)	nd	nd	nd	81.1
Chinese peas (boiled)	nd	93.1	nd	nd
Kala chana seeds (dry)	6.4	12.6	nd	61.3
Mung bean seeds (dry)	nd	nd	nd	nd
Mung bean sprouts (fd)	nd	nd	nd	nd
Clover sprouts (fd)	69.4	88.1	nd	5611.4
Alfalfa sprouts (fd)	nd	nd	nd	720.1

Note: nd = not detected; fd = freezedried (60 to 90% water loss); trace = < 1.

CHAPTER 2
MATERIALS AND METHODS

Materials

Genistein, biochanin A, and daidzein were purchased from Indofine Chemical (Somerville, NJ), and coumestrol from Acros (Pittsburgh, PA). Dimethylsuphoxide (DMSO), tyrphostin 25, β-mercaptoethanol, diethyl pyrocarbonate (DEPC), and trypan blue dye were purchased from Sigma Chemical Co. (St-Louis, MO). The following reagents were obtained from Fisher Chemical Co. (Pittsburgh, PA) :paraformaldehyde, methanol, chloroform, SDS, guanidium thiocyanate, phenol, isopropanol, sarkosyl, sodium bicarbonate, agarose and ammonium persulfate. Ethanol was purchased from AAPER ALCOHOL (Shelbyville, KY). All the radioisotopes (^{33}PdCTP, and ^{3}H-thymidine) were purchased from ICN (Irvine, CA). The biospin 6 column for purification of probes was from BIO-RAD (Hercules, CA). Microarray membranes and required reagents were from Research Genetics (Huntsville, AL). BCA for protein assay was purchased from PIERCE (Rockford, IL) and the ECL kit was from Amersham Pharmacia (Piscataway, NJ). The cell cycle test and the APO-BRDU kits are from Pharmingen (San Diego, CA). The human prostate cancer cell lines LNCaP (androgen-responsive) and PC-3 (androgen-independent) have been obtained from American Type Culture Collection (ATCC) (Rockville, MD). In addition, powded media RPMI 1640 and Ham F12 were obtained from ATCC (Rockville, MD). Trypsin, penicillin/streptomycin (pen/strep) and L-glutamine are from GIBCO (Gaithesburg, MD). Fetal bovine serum is

from Hyclone (Logan, UT). Mouse monoclonal antibodies against human $p21^{Cip1}$, p27 and cyclin D were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against human cyclin A, and cyclin B were obtained from BD Transduction Laboratories (San Diego, CA). Mouse monoclonal antibodies against human p53 were from Oncogene Research Products (Cambridge, MA). The goat anti-mouse secondary antibodies were purchased from Bio-Rad (Hercules, CA).

Methods

Cell Culture and Treatments

LNCaP cells were maintained in 10% fetal bovine serum in RPMI-1640 medium supplemented with 100 units/ml penicilin-streptomycin and 2 mM L-glutamine. PC-3 cells were maintained in Ham's F12K medium with 2 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. Both cell lines were maintained in T175 flasks, at 37 °C in a humidified atmosphere of 5% CO_2. For all experiments, the cells were used between passages 18 and 30.

LNCaP and PC-3 cells were seeded on 100 mm culture dishes at 1 x 10^6 cells in 10 ml of their respective medium. After 24 hours of being plated, the cells were treated with genistein, biochanin A, coumestrol, tyrphostin 25 (except otherwise specified, 37, 74, 111, 148 and 185 μM media) or 0.1 % DMSO as control. The phytochemicals were dissolved in DMSO for desired concentrations. At the time of treatment, fresh medium was applied, and treatment was continuous.

Cell Viability Assay

LNCaP and PC-3 cells were treated as described above. At 48 hours, the number of viable cells was evaluated by using trypan blue exclusion assay. At each collection, the cells from each culture dish were trypsinized, and suspended in a 15 ml tube. The cells were centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet resuspended in 1 ml of medium. From this suspension, 50 µl of the solution were added to 450 µl of the trypan blue solution 1:1 (v:v) with PBS (0.4% trypan blue, 0.81% NaCl, 0.06% K_3PO_4, pH 7.3). Viable cells were counted, using an hemocytometer, under a light microscope.

Apoptosis Assay

The level of DNA fragmentation, based on the terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling (TUNEL) assay, was determined using the APO-BRDU™ kit from PharMingen. LNCaP and PC-3 cells were treated with 0.1% DMSO, genistein, or biochanin A at 10, 20, 30, 40 and 50 µg/ml (37, 74, 111, 148, 185 µM). After 48 hours treatment, the cells were washed, collected, and fixed in 1% formaldehyde in PBS (pH 7.4) on ice. The cell pellets were then centrifuged (1000 rpm, for 5 minutes) and resuspended in PBS. The cell suspensions were then put in ice-cold ethanol overnight at -20 °C. The following day, the cells were centrifuged (1000 rpm, 5 minutes), resuspended in PBS and centrifuged. The cell pellets were put in the reaction buffer which contains BrdUTP stock solution, TdT (terminal deoxynucleotidyl transferase), $CoCl_2$ and distilled H_2O. The cells were incubated in this solution. FITC-conjugated anti-BrdUTP monoclonal antibody was added to the solution, and the incubation then continued in the dark for 30 minutes at room temperature. The cells were

analyzed by flow cytometry with the green fluorescence of FITC being detected at 530 ± 20 nm.

[^{3}H]Thymidine Incorporation Assay

LNCaP or PC3 cells were plated at a density of 5 x 10^4 cells/well in 24-well culture plates. After 24 hours, the cells were treated with varying concentrations of specific chemicals, as described previously, followed by the addition of 4 μCi/well [^{3}H]thymidine and pulsed for another 16 hours. Cells were trypsinized by the addition of 500 μl/well trypsin-EDTA, and collected on glass fiber filters using a Brandel cell harvester. The incorporation of [^{3}H]thymidine was measured by liquid scintillation counting.

Analysis of Cell Cycle Phase Distribution

Cells were collected at 48 hours following treatment with the respective isoflavonoids. The cells were washed with phosphate-buffered saline 3X (PBS), trypsinized. The analysis of the DNA content was performed using the CycleTEST^{TM} PLUS DNA reagent kit, from Becton Dickinson. The cells were centrifuged for 5 minutes at 300 *g* at room temperature (20 to 25 °C), and then washed in buffer solution. The supernatants were discarded, and the concentration of the cells was brought to 1 x 10^6 cells/ml in the buffer solution. The cell membranes were dissolved with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting RNA with RNase, and stabilizing the nuclear chromatin with spermine. Propidium iodide was stoichiometrically bound to the clean, isolated nuclei which was then analyzed with a flow cytometer. Propidium iodide-stained nuclei emit fluorescent light primarily

at wavelengths between 580 and 650 nm. The resulting histogram quantitated the number of cells in specific phases of the cell cycle based upon DNA content. Analysis was performed using the CELL Fit software program.

Western Blot Analysis

After treatment with the isoflavones or DMSO, the cells (LNCaP or PC-3) were washed three times in cold PBS. They were then collected in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM each of EGTA, NaF, PMSF, Na_3VO_4, and 1 μg/ml each of aprotinin, leuptenin, pepstatin.) The lysed cells were kept on ice for 30 minutes and centrifuged at 12,000 rpm for 15 minutes at 4 °C to pellet debris. A protein determination was perfomed using BCA Protein Assay Reagent kit. Total cellular protein (30-40 μg) was separated by one-dimensional sodium dodecyl sulfate polyacrylamdide gel electrophoresis (10 or 12% SDS-PAGE) and transferred to nitrocellulose membranes (Burnette, 1981). The membranes were then probed for the specific immunoreactive proteins followed by a secondary antibody against the species in which the primary was raised. Controls for the specificity of the primary antibodies were included using a known positive control on the same gel, or using a blocking peptide or normal serum (from the originating species) on a duplicate gel. Visualization of the immunoreactive proteins was done by enhanced chemiluminescent detection where the bands were detected by autoradiography. Bands were quantitated by densitometry with the autoradiographs being scanned, using a laser scanner (UMAX Astra 1200S). The images were digitized using Scion software, and the integrated mean density of the pixels present within the bands of interest was used for subsequent calculations.

Isolation of RNA

LNCaP and PC-3 cells were grown in T175 flasks and treated with the isoflavonoids at 37 μM and 111 μM in 20 ml of medium, respectively. After 48 hours, RNA collection was performed according to a modified from protocol from the single-step method (Chomczynski and Sacchi, 1987). Isolation buffer contained 5.7 M guanidium isothiocyanate (which protected the RNA from endogenous and exogenous RNases), N-lauryl sarcosine and β-mercaptoethanol (which rapidly disintegrated cellular structures, dissociated nucleoprotein complexes and lead to rapid denaturation). RNases are inactivated almost immediately. The intact RNA was purified by a double phenol/chloroform extraction. RNA selectively partitions in the aqueous phase, free from DNA (at the interface) and protein (organic phase) and is concentrated by isopropanol precipitation. RNA samples were treated with DNase. To prevent degradation of RNA, water and glassware were treated with 0.05% diethyl pyrocarbonate (DEPC). The yield and purity of RNA were assessed by spectrometry and by formaldehyde-agarose electrophoresis and ethidium bromide staining.

Microarray DNA Analysis

A human prostate-specific microarray membrane (5 cm x 7 cm) from Research Genetics was used which contained over 3,500 sequences that have been shown to be expressed in normal and malignant prostate tissue. Approximately 2,000 of these genes are known. See Chapter 5 for a more detailed description of the membrane.

For prehybridization, 0.5% SDS was heated to boiling. The membrane was washed in the heated 0.5% SDS solution for 5 minutes with gentle agitation to remove any residuals from the membrane. The membrane was then put in a hybridization roller

tube (35 x 150 mm) with the DNA side facing the interior of the tube. Ten ml of hybridization solution was added, as well as blocking reagents (10 µl cot-1 DNA and 10 µl poly-dA) provided by Research Genetics. The tube was vortexed thoroughly and the membrane was prehybridized for 2 hours.

For labeling the RNA, 1 µg of total RNA, suspended in 8 µl of DEPC water, was mixed with 2 µl of Oligo dT in a 1.5 ml microcentrifuge tube. The mixture was placed at 70 °C for 10 minutes, and briefly chilled on ice for 2 minutes. The following was added: 1 µl of DTT, 1.5 µl dNTP mixture, 1.5 µl reverse transcriptase and 10 µl of ^{33}P dCTP. The mixture was pulse centrifuged, and incubated for 1.5 hours at 37 °C. The probes were purified by passage through a column.

For hybridization, the purified probes were denatured by boiling for 3 minutes. The probes were pipetted in the roller tube containing the membrane and the prehybridization solution. The membrane was hybridized for 12-18 hours overnight at 42 °C. The following day, the membrane was washed twice in 2X SSC, 1% SDS at 50 °C for 20 minutes, and once with 0.5X SSC, 1% SDS at room temperature for 15 minutes. The membrane was moistened with deionized water and wrapped in plastic wrap. The membrane was placed in a cassette with a phosphor imaging screen.

RNA with ^{33}P labelled nucleotides from cells treated with isoflavonoids or DMSO were hybridized with individual membranes. The analysis and comparison of RNA expression levels was made from phosphor imaging data using the Pathways™ software. This program is a Windows95/Windows NT analysis software used in conjunction with GeneFilters. A built-in database facilitates archiving of both raw images and fitted data

Data analysis was achieved by using several different programs. Arbitrary intensity data from all array spots were obtained from imager scans using Pathways™ software (Research Genetics, Huntsville, AL). Numerical data analysis was performed with Microsoft Excel 97. Clustering of gene expression patterns was done by using the program Cluster and Treeview written by Michael Eisen from Stanford University (Eisen *et al.*, 1998). See Chapter 5 for a more in-depth description of the data analysis.

Animal Studies

Mice. Male *nu/nu* athymic nude mice were used at 6 to 8 weeks old. These mice are immunocompromised and are suitable recipients for human prostate cancer cells. They were housed under sterile conditions in autoclaved cages with filter bonnets in laminar flow units and fed sterilized pellets. The pelleted diets were sterilized by irradiation rather than autoclaving, which may destroy heat sensitive ingredients. All mice were on a casein-based diet (soy-free) 5K96CI from Purina Mills (Richmond, IN). See Chapter 4 for the designs of the experimental models.

Immunohistochemical analysis. Tumors from xenograft athymic nude mice were cut into pieces no larger than 3 mm in thickness. The tissue was fixed in 10% formalin at room temperature for 8-12 hours followed by 9 hour processing in increasing concentrations of alcohol (50 to 100%), xylene and paraffin. Following infiltration of the tissue with paraffin, tissues were embedded in paraffin blocks. Sections (4 μm) were picked up on poly-l-lysine slides (3 slides per mouse) and dried upright overnight at room temperature. The sections were deparaffinized in 2 changes of xylene for 10 minutes each, afterwhich the slides were transferred to 2 changes of 100% alcohol for 2 minutes each. Endogenous peroxidase activity in the sections was blocked with 3% H_2O_2 in

methanol. The slides were then rinsed with water, and pretreated with 10 mM citrate buffer and microwaved. The slides were rinsed 2-3 times, and excess buffer was wiped out from around the specimen. For the determination of the level of cells undergoing mitosis, one slide per mouse was stained with hematoxylin and eosin, and analyzed by light microscopy. Blocking with 5% normal serum (from the species the secondary antibody is made) was performed. Primary antibodies against specific immunoreactive proteins (CD34) were applied to cover the tissue, followed by incubation at 4 °C overnight in a humid chamber. Control for the specificity of staining was one tissue section treated with PBS. The slides were rinsed in PBS, and treated with biotinylated secondary antibodies and incubated at room temperature for 30 minutes. After being rinsed, the slides were treated with streptavidin/HRP and incubated at RT for 30 minutes. The slides were rinsed in PBS, dried and treated with DAB solution for 5 minutes. After draining off excess DAB, the slides were rinsed in water and counterstained with hematoxylin and dilute ammonia water. A light microscope was used to visualize staining for CD34, hematoxylin and eosin (H&E). For the CD34 staining, 2 fields (1.56 mm^2/field) were surveyed at a magnification of 20X per slide per animal. For the H&E staining, 6 fields were surveyed per slide per animal.

Data Analysis

All experiments were repeated three times unless otherwise stated. For analysis of scanned images, control lanes were standardized to 100% and all treatments were assessed relative to controls for each individual experiment. Results from the three experiments are represented as mean ± standard error of the mean and the Student's t-test used to analyze the data and to determine p-values. Two-way ANOVA analysis was

performed to assess the significance of the effects of the isoflavonoid treatment in the xenograft nude mice. Statistical analyses were performed using Microsoft Excel and Prism 2.01 softwares.

CHAPTER 3
EFFECTS ON CELL PROLIFERATION

Introduction

During development, the body grows through multiplication of cells. In this process, a cell duplicates its content and gives rise to two identical daughter cells. Cellular division is a highly regulated process, which is responsive to the environment and the specific needs of the whole organism. In the early stage of development, cell multiplication far exceeds cell death, such that the human body increases in size. Distinct organs adopt well-defined shapes and functions (Evan and Littlewood, 1998) through cell death. For example, fingers and toes are formed from the death of cells in the interdigital tissue (Kerr *et al.*, 1972; Novack and Korsmeyer, 1994). In addition, formation of the T and B cell repertoires of the immune system is dependent on both negative selection by apoptosis (elimination of non-reactive or self-reactive cells) and on positive selection (Cohen, 1991). However, the process of cell birth and cell death reaches a balance, with a few exceptions depending on the cell type. Notably, certain white blood cells and intestinal cells undergo cell death only a few days after being generated. In contrast, healthy liver cells seldom die, neurons rarely divide and experience little or no replacement (Lodish *et al.*, 1996).

This highly regulated machinery for cell multiplication can be altered, with cellular mutation or improper signal transduction, wherein the overall steady state between cell birth and cell death is lost (Lopez-Saez *et al.*, 1998). A cell can continue to

grow and divide regardless of its environment and the need of the organism. The resulting daughter cells will inherit the propensity to divide without responding to regulation, and eventually leading to clones of cells that can expand indefinitely. During the early phase of the growth of the initiated clones, this neoplasm will remain clinically undetectable until it reaches a critical mass detectable as a tumor (Hanahan and Weinberg, 2000; Heppner and Miller, 1998).

The goals of most cancer research are two-fold. First, it is to understand and to identify alterations in the regulation machinery of cell multiplication in cancer cells. Second, it is to develop ways to exploit the weakness of the cancer cells, in order to halt their growth and division, or to reclaim control of the cell multiplication. This project focuses on the second aspect of cancer research that is to investigate the effects of the isoflavonoids on the proliferation of human prostate cancer cells, or more specifically on their ability to induce cell cycle arrest and/or apoptosis.

Regulation of the Cell Cycle

Progression of the cell cycle is carefully coordinated by a series of events that culminate in DNA synthesis and cell division. Phosphorylation of selected proteins by cyclin-dependent kinases is the driving engine of a successful cell cycle (Morgan, 1995). The highly conserved nature of this machinery in different eukaryotic organisms emphasizes its early origin and its importance for life (Beach *et al.*, 1982; Gautier *et al.*, 1988). The control of multiplication relies on accelerating and braking mechanisms, which act on the engine driving the cell cycle. There are many checkpoint pathways that can prevent important cycle transitions (e.g., initiation of proliferation, replication, mitosis) until the integrity of the DNA and other cellular conditions allow for progression

(Zetterberg *et al.*, 1995). These checkpoint pathways are able to transduce signals regarding the adequacy of initiating or continuing proliferation for a cell at a particular time, under a given set of external and internal conditions. Crucial components of these pathways are regulatory proteins encoded by some of the checkpoint genes that evaluate the final balance of mitogenic and antimitogenic pathways reaching them. When the balance is negative, they prevent cell cycle intiation or it progression. When the balance is positive, the regulatory proteins allow progression in the cell cycle (Lopez-Saez *et al.*, 1998).

The cell cycle consists of four phases G1, S, G2, and M (Figure 3-1). In G1, cells integrate growth promoting and/or growth inhibiting environmental cues to determine whether they will advance through the mitotic cell cycle or into quiescence (Pardee, 1989; Sherr, 1994). Cyclin dependent kinases (CDKs) dictate transitions into all the phases of the cell cycle (Nigg, 1995; Peeper *et al.*, 1994). The activity of these kinases is regulated at many levels, including (a) both inhibiting and stimulatory phosphorylation events, (b) binding to a cyclin, and (c) binding to CDK inhibitors (Hunter and Pines, 1994; Morgan, 1995). Cell cycle progression in G1 is regulated by the activity of cyclin D and E and their associated CDKs. D-type cyclins are the earliest of the cyclins to accumulate in the cell cycle, and once produced, they associate with and promote the activation of their CDK partners, CDK4 or CDK6 (Sherr, 1995a, 1996). Subsequent to CDK4(6)-cyclin D activation, cyclin E accumulates and activates CDK2 for progression through G1. Cyclin A then associates with both CDK2 (S phase) and CDK1 (late S and G2). Cyclin B needs to associate with CDK1 in order for the cell to proceed in G2, and to enter the M phase. Cyclin A-associated kinases are required for progression through

late G1 and completion of S phase (Peeper *et al.*, 1994; Resnitzky *et al.*, 1995; Sherr, 1995b). In addition, CDKs are regulated by cyclin-dependent kinase inhibitors (CDKIs). These molecules attenuate the kinase activity of CDKs by binding directly with active CDK-cyclin complexes or by competing with cyclin for binding to CDKs (Hunter and Pines, 1991). The CDKIs can be divided into two families, the INK4 family (p16, p15, p18 and p19), which act early in G1 to inhibit the activity of CDK4 and CDK6, and the CIP family ($p21^{Cip1}$, p27 and p57), which inhibit all CDK-cyclin complexes (Sherr and Roberts, 1995). The main substrate of the G1 cyclin-associated kinases is the retinoblastoma tumor suppressor protein, RB (Sidle *et al.*, 1996). Prior to its phosphorylation by G1 CDKs, RB is believed to prevent progression to S phase by sequestering key proteins required for DNA synthesis such as E2F (Beijersbergen and Bernards, 1996). E2F has been reported to regulate the expression of a number of genes required for cell cycle progression (DeGregori *et al.*, 1995; Slansky and Farnham, 1996). Binding of RB to E2F results in repression of E2F-responsive genes, thus preventing S phase initiation (Beijersbergen and Bernards, 1996; DeGregori *et al.*, 1995; Slansky and Farnham, 1996; Wang *et al.*, 1994).

Apoptosis

A Cell Cycle Phenomenon

The cell cycle is the highly conserved innate mechanism by which eukaryotic cells replicate themselves. Apoptosis is the highly conserved innate mechanism by which eukaryotic cells commit suicide. At first look, the biological endpoint of these two processes could not be more different (death and life). However, apoptosis can be considered as a process intimately linked to the cell cycle (Lopez-Saez *et al.*, 1998;

Meikrantz and Schlegel, 1995). There are several lines of evidence indicating that apoptosis is a cell cycle phenomenon. First, within the organism, apoptosis is almost exclusively found in proliferating tissues (Lopez-Saez *et al.*, 1998; Wyllie *et al.*, 1980). Second, artificial manipulation of the cell cycle can either prevent (by elimination of regulatory checkpoint proteins) (Baker *et al.*, 1990; Isaacs *et al.*, 1991) or potentiate (by overexpression of certain negative cell cycle regulatory proteins) apoptosis (Clarke *et al.*, 1994; Lowe *et al.*, 1993), depending on the point of arrest.

In addition, many oncogenes that promote cell cycle progression also induce apoptosis. The mammalian cellular protein c-Myc exhibits duality of function. It can act as a potent inducer of both cell proliferation and apoptosis (Ucker, 1991). The c-Myc protein is a short-lived, sequence specific, DNA-binding protein that resembles a transcription factor of the basic helix-loop-helix leucine zipper (bHLH-LZ) class (Evan *et al.*, 1995). Mutational mapping of c-Myc shows complete coincidence between those domains involved in mitogenesis, apoptosis, and transcriptional activation; these regions are the amino-terminal transactivation domain and the carboxy-terminal DNA-binding and dimerization domains (Amati *et al.*, 1993; Evan *et al.*, 1995).

Other key tumor suppressor proteins, like p53, exert direct effects both on cell viability and on the cell cycle progression. p53 either enforces cell cycle arrest or triggers apoptosis. Which of these two alternative fates occurs in any particular instance appears to depend on a number of undefined factors, including cell type, nature and intensity of insult, cytokine status and experimental paradigm. The high frequency with which p53 is functionally inactivated in human tumours (50%) attests to the critical role that p53 exerts in limiting neoplastic expansion (Hollstein *et al.*, 1991; Vogelstein, 1990). p53 is now

widely recognized as a transducer of genome damage into growth arrest and/or apoptosis, thus preventing the propagation of potentially mutant cells together with the neoplastic risk they present. The p53 protein can act as a transcription factor, and most evidence indicates that it induces growth arrest by the modulation of specific target genes (Evan and Littlewood, 1998). Most prominent of these is the induction by p53 of the gene encoding the Cdk inhibitor $p21^{Cip1}$, whose action arrests cells in late G1 (el Deiry *et al.*, 1994; Harper *et al.*, 1993; Xiong *et al.*, 1993). The potency with which so many mitogenic oncogenes induce apoptosis argues persuasively for a direct mechanistic link between the molecular mechanisms that mediate cell cycle and cell death.

Mechanism of Programmed Cell Death

Apoptotic cells are associated with a number of distinct morphological changes. These physical changes include a loss of cell volume (condensation), margination of the chromatin and its redistribution against the nuclear envelope (pyknosis), and dissociation of the dying cell from its healthy neighboring cells. Cytoplasmic organelles are maintained intact until the final stages of cell death, a process marked by the formation and release of plasma membrane-bound vesicles (apoptotic bodies) containing the cellular constituents of the dying cell (Kerr *et al.*, 1972). One hallmark attributed to apoptotic cells is the loss of genomic DNA integrity following endonuclease-mediated fragmentation. A primary indicator of this event is the generation of "DNA ladders" from the cleavage of DNA into 180-base pair oligonucleosomal multiples (Wyllie, 1980).

The regulated destruction of a cell is a complicated process, which requires the cell to participate in its own demise (Arends *et al.*, 1994). The decision to die is tightly controlled, and the likelihood of a cell activating its self-destruction program is

influenced by the activity of many genes. Most of the morphological changes are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These death proteases are homologous to each other and are part of a large protein family known as caspases (Alnemri *et al.*, 1996). Activation of caspases does not lead in the wholesale degradation of cellular proteins (Nicholson, 1999). Rather, caspases selectively cleave a restricted set of target proteins, usually at one, or at most a few positions in the primary sequence (always after an aspartate residue) (Garcia-Calvo *et al.*, 1999). While caspases tend to render target proteins inactive, they can also activate proteins, either directly, by cleaving off a negative regulatory domain, or indirectly by inactivating a regulatory subunit (Hengartner, 2000). For example, the DNA ladder nuclease (now known as caspase-activated DNase, or CAD) pre-exists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD (Nagata, 2000). Activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit (Enari *et al.*, 1998; Liu *et al.*, 1997). Caspase-mediated cleavage of specific substrates also explains several other characteristic features of apoptosis, including nuclear shrinking, budding, loss of cell shape and blebbing (Buendia *et al.*, 1999; Rao *et al.*, 1996).

Results

Effects on Cell Viability

Treatment with the isoflavonoids caused a dose-dependent decrease in the number of viable cells in LNCaP cells after 48 hours (Figure 3-2A). Genistein and biochanin A had the most potent inhibitory effects compared to daidzein and coumestrol. The IC_{50} for genistein and biochanin A was 63 μM. PC-3 cells were similarly affected by the

isoflavonoids, with cell viability decreasing in correlation with increasing doses of the phytochemicals after 48 hours (Figure 3-2B). As in LNCaP cells, genistein and biochanin A were the most potent in PC-3 cells, with IC_{50} values being very similar to those in LNCaP cells.

Induction of DNA Fragmentation

The TUNEL assay was used to determine the level of DNA fragmentation which is indicative of apoptosis. Genistein and biochanin A increased the percentage of apoptotic cells in a dose-dependent manner after 48 hours in LNCaP cells (Figure 3-3A). On the other hand, PC-3 cells exhibited a very low level of DNA fragmentation after treatment with either genistein or biochanin A, under similar conditions as in the LNCaP cells (Figure 3-3B). At the highest concentration, less than 3% of the PC-3 cells exhibited DNA fragmentation.

Effects on DNA Synthesis

The isoflavonoids blocked de novo DNA synthesis in a dose-dependent fashion in LNCaP cells after 48 hours (Figure 3-4A). This was determined by measuring the effects on ^{3}H-thymidine incorporation. Daidzein and coumestrol showed strong inhibitory effects, at 185 μM but not at 37 μM, in this assay. Genistein and biochanin A were, however, the most potent inhibitors of de novo DNA synthesis. At 37 μM, both of these isoflavonoids completely blocked incoporation of ^{3}H-thymidine. This concentration of genistein and biochanin will be used in susbsequent experiments in LNCaP cells. DNA synthesis was also decreased in a dose-dependent manner by the isoflavonoids in PC-3 cells (Figure 3-4B). The inhibitory curves in this prostate cancer cell line were shifted to

the right. Complete blockage of ^{3}H-thymidine incorporation was achieved at 111 μM. This concentration of genistein and biochanin will be used in susbsequent experiments in PC-3 cells.

Effects on Cell Cycle Progression

Genistein, at the concentration of 37 μM in the LNCaP cells, increased the number of cells in the G1 phase from 73.3% to 78.5% ($p = 0.018$), and in the G2/M phase from 8.9% to 14.7% ($p = 0.003$) (Figure 3-5) (Figure 3-7A). In addition, the number of cells in the S phase decreased from 17.8% to 6.7% with genistein ($p = 0.002$). This is characteristic of a G2/M accumulation in the LNCaP cells. Biochanin A, at 37 μM, increased the number of cells in the G1 phase from 73.3% to 86.2% ($p = 0.004$) (Figure 3-5) ((Figure 3-7A). Like genistein, biochanin depleted the percentage of cells in the S phase from 17.8% to 7.2% ($p = 0.001$), with no change in G2/M. Biochanin A appears to elicit a G1 arrest in the LNCaP cells. Daidzein had a less pronounced effect of the cell cycle phase distribution, with an increase in the percentage of cells in the G1 phase from 73.3% to 77.5% ($p = 0.036$), and a decrease in the percentage of cells in the S phase from 17.8% to 13.7% ($p = 0.028$) (Figure 3-5) ((Figure 3-7A).

In the PC-3 cells, the isoflavonoids affected the cell cycle phase distribution in a different fashion (Figure 3-6) (Figure 3-7B). Genistein, at its the concentration of 111 μM in the PC-3 cells, decreased the percentage of cells in the G1 phase from 67.3% to 44.4% ($p = 0.009$), but increased the number of cells in the S phase and G2/M phase, from 18.7% to 24.3% ($p = 0.025$), and from 14.1% to 31.4% ($p = 0.011$), respectively. The cells accumulated in the S and G2/M phases. Biochanin A, at 111 μM, decreased the number of cells in the G1 phase from 67.3% to 37.55% ($p = 0.002$), which is markedly

different than the increase observed in LNCaP cells. Furthermore, in pc-3 cells, biochanin significantly increased cells in both S and G2/M phases, from 18.7% to 36.34% ($p = 0.013$), and from 14.1% to 26.1% ($p = 0.002$), respectively. Thus in PC-3 cells, biochanin had similar effects as genistein, with cells exiting G1 and accumulating in the S and G2/M phases. Daidzein had similar effects on the cell cycle, but were less pronounced, with a decrease in the number of cells in the G1 phase from 67.3% to 52.2% ($p = 0.014$), and an increase in the S and G2/M phase, from 18.7% to 30% ($p = 0.016$), and from 14.1% to 17.7% ($p = 0.025$), respectively.

Effects on Cyclins

Cyclin A. In LNCaP cells, after 48 hours, genistein and biochanin A, at 37 μM, significantly decreased cyclin A protein expression by 33% ($p = 0.004$) and 25% ($p = 0.029$), respectively (Figure 3-8A). Daidzein and coumestrol had little effect. In PC-3 cells, after 48 hours, cyclin A protein expression was significantly decreased only biochanin A at 111 μM, by 60 % compared to control ($p = 0.002$) (Figure 3-8B).

Cyclin B. In LNCaP cells, after 48 hours, genistein and biochanin A, at 37 μM, significantly decreased cyclin B protein expression by 90 % ($p < 0.01$) (Figure 3-9A). Daidzein and coumestrol, however, had no significant effects on the level of cyclin B protein. In PC-3 cells, after 48 hours, cyclin B protein expression was decreased only by biochanin A at 111 μM, by 30 % compared to control ($p = 0.017$) (Figure 3-9B). Genistein increased the protein level of this cell cycle regulator by 35% ($p = 0.044$). Daidzein and coumestrol also increased the protein level of cyclin B, by 26% ($p = 0.031$) and 78% ($p = 0.008$), respectively.

Cyclin D. The progression through the cell cycle is dependent on the activity of a number of kinases. These kinases, in turn, need to be associated with specific proteins (cyclins) to be active. In the G1 phase, the presence of cyclin D is important for the activity of the kinases in this phase. Since PC-3 cells were not arrested at G1 with either of the chemicals, the effects of the isoflavonoids on cyclin D were investigated only in LNCaP cells. After 48 hours, genistein and daidzein, at 37 μM, decreased significantly the protein expression level of cyclin D in LNCaP by 8% ($p = 0.046$) and 12% ($p = 0.047$) respectively (Figure 3-10). Biochanin and coumestrol had no significant effects on the level of cyclin D.

Effects on Cyclin-Dependent Kinase Inhibitors

$p21^{Cip1}$. In LNCaP cells, after 48 hours genistein at 37 μM, increased $p21^{Cip1}$ protein expression by 79% ($p = 0.048$) (Figure 3-11A). Biochanin A, however, lowered $p21^{Cip1}$ protein expression by 50% ($p = 0.042$) (Figure 3-11A). Coumestrol decreased the level of $p21^{Cip1}$ by 30% ($p = 0.005$). Daidzein had no effect. In PC-3 cells $p21^{Cip1}$ protein expression was induced by genistein at 111 μM ($p = 0.032$) (Figure 3-11B). On the other hand, the other isoflavonoids including biochanin A did not elicit the induction of $p21^{Cip1}$ (Figure 3-11B).

p53. The tumor suppressor protein p53 is a very important cell cycle regulatory protein, which can induce apoptosis or arrest cells in the G1 phase depending on the external and internal cues. Since PC-3 cells do not have a functional p53, the effects of the isoflavonoids were investigated only in LNCaP cells. After 48 hours, the isoflavonoids at 37 μM, including genistein and biochanin A had no effects on the protein expression level of p53 in LNCaP cells (Figure 3-12).

p27. The cell cycle regulatory protein p27 can act as a cyclin-dependent kinase inhibitor at all phases of the cell cycle. After 48 hours, the isoflavonoids at 37 μM, including genistein and biochanin A had no effect on the protein expression level of p27 in LNCaP cells (Figure 3-13). In PC-3 cells, immunoreactive p27 protein was not detectable.

Effects of Tyrosine Kinase InhibitorTyrphostin 25

The antiproliferative effects of the isoflavone genistein has been described to be due to its tyrosine kinase inhibition activity. Genistein, however, exhibits many modes of action (see Chapter 1) that may explain its antiproliferative effects. In order to test the role of tyrosine kinase inhibition in the effects observed in the prostate cancer cells with treatment of genistein, the effects of another specific tyrosine kinase inhibitor tyrphostin 25 were evaluated in the prostate cancer cells. In addition, the effects of tyrphostin are compared to those of the isoflavone daidzein, which is sometimes used as a negative control for tyrosine kinase inhibitors.

Cell viability. The proliferation of LNCaP cells, after being treated for 48 hours with tyrphostin 25, decreased in a dose-dependent manner with an IC_{50} value of 250 μM (Figure 3-14A). The inhibitory effect of tyrphostin is, however, less pronounced than genistein and daidzein, with IC_{50} values of 63 μM and 111 μM, respectively (Figure 3-14A; Table 3-1). PC-3 cells were also sensitive to the tyrosine kinase inhibitor tyrphostin 25, after 48 hours (Figure 3-14B). The number of viable cells decreased in a dose-dependent manner with the inhibitor with an IC_{50} value of 250 μM. The antiproliferative effects were, as in LNCaP cells, much less pronounced than with genistein and daidzein with, with IC_{50} values of 63 μM and 148 μM, respectively (Figure 3-14B; Table 3-1).

DNA synthesis. DNA synthesis, as measured by ^{3}H-thymidine incorporation in LNCaP cells after 48 hours, is inhibited in a dose-dependent manner by tyrphostin 25, a protein tyrosine kinase inhibitor with an IC_{50} value of 45 μM (Figure 3-15A). This effect was much stronger than daidzein which had a IC_{50} value of 92.5 μM, but less than genistein with IC_{50} value of 15 μM (Figure 3-15A). Tyrphostin exhibited similar inhibition in the PC-3 cells after 48 hours, with a IC_{50} value of 125 μM, whereas genistein and daidzein had IC_{50} values of 56 μM and 104 μM, respectively (Figure 3-15B). Complete blockage of incorporation of radiolabeled thymidine was observed in both cell lines with tyrphostin 25 at 250 μM (Figure 3-15B).

Cyclin B protein level. Western immunobloting showed that, like daidzein, tyrphostin 25,at 250 μM after 48 hours, had no significant effects on cyclin B protein expression in LNCaP cells, whereas genistein significantly decreased the level of cyclin B by 90% (Figure 3-16A). In PC-3 cells, cyclin B protein expression was lowered by 22% with tyrphostin ($p = 0.033$), whereas genistein and daidzein significantly increased the level by 35 and 26%, respectively (Figure 3-16B).

$p21^{Cip1}$ protein level. Like daidzein in LNCaP cells, tyrphostin (250 μM) had no effects on protein expression of $p21^{Cip1}$. Genistein, however, significantly increased the level by 2-fold (Figure 3-17A). In PC-3, $p21^{Cip1}$ protein expression was significantly lowered by 20% with tyrphostin, but the protein level was significantly increased by genistein by 2-fold (Figure 3-17B). Daidzein had no significant effects.

Discussion

The two human prostate cancer cell lines used in this study display characteristics of a clinically early stage (LNCaP, androgen-responsive) and a clinically

late stage (PC-3, androgen independent) of prostate cancer (Taplin *et al.*, 1995). Genistein and biochanin A can affect these cells at both stages. The cell viability, and de novo DNA synthesis is inhibited in a dose-dependent manner in both cancer cell lines by genistein and biochanin after 48 hours. The inhibition of proliferation of human prostate LNCaP cancer cells by genistein and daidzein has been reported by others (Onozawa *et al.*, 1998). In addition PC-3 cells have been described to have decreased proliferation with treatment of genistein, biochanin and daidzein (Hempstock *et al.*, 1998; Davis *et al.*, 1998; Kyle *et al.*, 1997; Onozawa *et al.*, 1998). These antiproliferative effects have also been observed in other cancer cell lines, such as human breast carcinoma cell lines (Peterson and Barnes, 1991; Shao *et al.*, 1998), gastrointestinal cancer cell lines (Yanagihara *et al.*, 1993), and leukemia cells (Bergamaschi *et al.*, 1993).

In the present study, the level of apoptosis in LNCaP cells, as determined by the level of DNA fragmentation, increased with increasing concentrations of genistein and biochanin. This induction of apoptosis in LNCaP cells has been well-described with treatment of genistein and daidzein (Onozawa *et al.*, 1998), and in a PC-3-M metastatic variant cell line with genistein (Kyle *et al.*, 1997). Other human cancer cell lines also undergo programmed cell death with the isoflavonoids, including leukemic cell lines with genistein (Bergamaschi *et al.*, 1993), and stomach cancer cell lines with genistein and biochanin (Yanagihara *et al.*, 1993; Kyle *et al.*, 1997).

PC-3 cells, however, in this study did not respond similarly with increasing concentrations of the isoflavones. When the level of fragmented genomic DNA was determined by TUNEL assay, PC-3 cells showed a very low level with genistein and biochanin, even at the highest concentration of 185 μM at which cells were clearly

nonviable. The PC-3 cells appear to be resistant toward induction of apoptosis. The present study is the first to report this differential effect of the isoflavones in LNCaP and PC-3 cells. However, a similar differential effect between the responses of the two cell lines has been observed with treatment of resveratrol, where this compound induces apoptosis in LNCaP cells, but not in PC-3 (Hsieh and Wu, 1999).

Since PC-3 cell viability decreased in a dose-dependent fashion with the phytochemicals, it would appear that the results of the two assays are contradictory. However, consideration must be given to the trypan blue exclusion assay, which is based on the fact that non-viable cells are leaky and can accumulate the dye with much more ease than their viable counterparts (Arends *et al.*, 1990). After 48 hours, PC-3 cells may have become leaky with exposure to the isoflavonoids, but their genomic DNA may not have been degraded by the activated endonucleases at the time point being investigated. It is also possible that a distinct population of PC-3 cells had undergone apoptosis earlier than 48 hours at the time when the level of DNA fragmentation was assessed. The presence of such an apoptotic population would not have been detected in this study. In addition, PC-3 cells may undergo different modes of cell death, including necrosis (Arends *et al.*, 1994). One report, in fact, has described PC-3 cells undergoing necrosis rather than apoptosis with zinc treatment (Iguchi *et al.*, 1998). The resistance of PC-3 cells toward induction of apoptosis is also not surprising considering that their p53 protein is not functional, and the role of this tumor suppressor protein in the induction of apoptosis (Baker *et al.*, 1990; Hengartner, 2000).

Another possible mode of programmed cell death in PC-3 cells with the isoflavones is autophagy (Bursch *et al.*, 2000a). This type 2 nonapoptotic form of

programmed cell death is characterized by lysosomal degradation of cellular organelles, associated with an intact cytoskleton and a lack of DNA degradation, which will make this process negative for TUNEL staining (Bursch *et al.*, 2000b; Chi *et al.*, 1999). Furthermore, PC-3 cells have been shown to undergo cell death by autophagy with treatment of the peroxisome proliferator-activated receptor γ ligand, 15-$\Delta^{12,14}$-prostaglandin J_2 (Butler *et al.*, 2000), which was marked with an increase in S phase as observed in the present study with the isoflavones. Similar induction of autophagy in PC-3 cells has been described with treatment of 5-lipoxygenase inhibitors (Anderson *et al.*, 1998).

In the present study, genistein accumulated both LNCaP and PC-3 cells in the G2/M phase of the cell cycle, which is in agreement with what has been observed by others in these prostate cancer cells (Choi *et al.*, 2000; Davis *et al.*, 1998). This effect on cell cycle progression has been reported as well in a number of other cancer cell lines, including leukemic cells (Spinozzi *et al.*, 1995; Traganos *et al.*, 1992; Spinozzi *et al.*, 1994), and gastric cancer cells (Matsukawa *et al.*, 1993; Traganos *et al.*, 1992). Furthermore, the present study is the first to report that biochanin accumulated LNCaP cells in the G1 phase, and PC-3 cells in the G2/M phase, which is contrary to an earlier report that biochanin has no effects on cell cycle phase distribution in human bladder cancer cells (Su *et al.*, 2000). The inability for the PC-3 cells to undergo a G1 arrest may be due in part to the non-functional p53 in this cell line. This tumor suppressor protein is instrumental in a G1 arrest of the cell cycle (el Deiry *et al.*, 1994; Evan and Littlewood, 1998). At least in LNCaP cells, the structurally similar isoflavonoids genistein and

biochanin appear to act through different pathways by which they affect the cell cycle progression.

Future experiments should include transfection of PC-3 cells with a wild-type p53 to test the sensitivity of these cells toward treatment of the isoflavonoids. If a G1 arrest is observed with treatment of biochanin in these transfected PC-3 cells, it would suggest the effect of biochanin on the G1 phase is p53-dependent. In the LNCaP cells, both genistein and biochanin decreased the percentage of cells in the S phase after 48 hours. This effect supports the data from the ^{3}H-thymidine incorporation assay where at 37 μM complete inhibition was observed, thus indicating decreased de novo DNA synthesis. In the PC-3 cells, however, the flow cytometry data did not agree with the ^{3}H-thymidine incorporation assay. Both phytochemicals (111 μM) were found to decrease the incorporation of the labeled thymidine while increasing the number of cells in the S phase. This indicates that the PC-3 cells, according to their DNA content, may be accumulating in the S phase without active DNA synthesis. It is also possible that the isoflavones are blocking the transport of the ^{3}H-thymidine into the cells rather than inhibiting incorporation. Several chemicals, including D-threo-1-phenyl-2-decanoylamino-3-morpholino- 1-propanol (PDMP) and dipyridamole, are known to block the transport of thymidine into the cells, rather than inhibiting incorporation (Griner and Bollag, 2000; Smith *et al.*, 2000). Consequently, the PC-3 cells may be incorporating unlabeled thymidine through the thymidine salvage pathway.

The G2/M cell cycle accumulation in LNCaP by genistein, and in PC-3 by both genistein and biochanin may be partly explained, in the present study, by the decrease in the level of proteins of the positive cell cycle regulator cyclin B, and the increase in the

level of the negative regulator $p21^{Cip1}$. Similar alteration in the protein expression of these cell cycle regulators has been reported with genistein treatment in human prostate cancer cells (Choi *et al.*, 2000; Davis *et al.*, 1998). The increase in $p21^{Cip1}$ protein level by genistein implicates a p53-independent induction since PC-3 cells have a mutated non-functional p53. Future experiments should include blocking the expression of $p21^{Cip1}$ with mRNA antisense, or overexpressing a dominant negative form of $p21^{Cip1}$ in genistein-treated cells to test the role of the induction of this cyclin-dependent kinase inhibitor in the mechanism of action of genistein on the cell cycle phase distribution.

In the present study, biochanin appears to lower the level of $p21^{Cip1}$ in LNCaP cell line, which is the first report of this kind. This decrease is somewhat counterintuitive considering that both cell lines exhibit cell cycle arrest with biochanin, and that a decrease in $p21^{Cip1}$ can promote progression through the cell cycle (Harper *et al.*, 1993; Xiong *et al.*, 1993). Biochanin, however, does affect other cell cycle regulatory proteins involved in the control of progression. For example, the present study shows for the first time that cyclin B protein level is barely detectable in LNCaP cells with biochanin. Without the association of cyclin B with cdk1, cells accumulate at the end of G2, and are unable to enter the M phase (Peeper *et al.*, 1994). In addition, cyclin A protein expression level was significantly reduced by both genistein and biochanin in LNCaP, and by biochanin in PC-3 cells. This effect on cyclin is first observed in the present study. Since many regulatory proteins are involved in the control of the cell cycle, future experiments should test the importance of the two cyclins in the effects of biochanin by overexpressing them in the two cell lines. Moreover, the transfected cells should be treated with biochanin in order to evaluate whether the effects remain the same.

Genistein has been well described for its ability to inhibit tyrosine kinase enzyme activity of purified receptors, especially EGF-R (Akiyama *et al.*, 1987). Other purified receptor tyrosine kinases inhibited by genistein include c-src and v-abl (Akiyama *et al.*, 1987). Genistein does not, however, inhibit all receptor tyrosine kinases, such as the insulin receptor (Abler *et al.*, 1992). Furthermore, platelet-derived growth factor and nerve growth factor receptors are able to transmit mitogenic signals in whole cells despite genistein concentrations over 111 μM (Abler *et al.*, 1992; Davidai *et al.*, 1992). In the literature, a concentration of genistein and biochanin which is comparable to what was used in the present study, that inhibits proliferation of prostate cancer cells, including LNCaP, was reported to inhibit the activity of EGF-R tyrosine kinase (Peterson and Barnes, 1993). Futhermore, others have reported difficulty in detecting a decrease in the tyrosine phosphorylation of discrete proteins after genistein treatment (Peterson, 1995). In the present study, while the EGF-R specific tyrosine kinase inhibitor tyrphostin 25 decreased, in a dose-dependent manner, cell viability and de novo DNA synthesis in both prostate cancer cell lines, the IC_{50} values for these endpoints were higher than these for genistein and biochanin. The present study is the first to investigate the effect of tyrphostin 25 on cell cycle regulatory proteins. Tyrphostin was not able to affect the protein level of cyclin B and $p21^{Cip1}$ in the same manner as genistein and biochanin A. These data suggest that the antiproliferative effects of genistein observed in this study likely involve more than solely inhibition of EGF-R tyrosine kinase.

While there is no defined or unique receptor for the isoflavonoids, the various effects observed at the concentration range used in this study parallel those at which genistein as been shown to inhibit topoisomerase activity (Constantinou *et al.*, 1990;

Markovits *et al.*, 1989; Okura *et al.*, 1988). Inhibition of topoisomerase by other inhibitors has been associated with blockage of the cell cycle progression (Del Bino *et al.*, 1991; Dubrez *et al.*, 1995; Russo *et al.*, 1994). Furthermore, the alteration in the expression of cell cycle regulatory proteins has been observed in the inhibition of topoisomerase activities (Deptala *et al.*, 1999; Janss *et al.*, 2001).

The present study uniquely showed the importance of the chemical structure of the isoflavone for the negative effects on cell proliferation. Among the isoflavonoids screened, genistein and biochanin are the ones that have been consistently found to be the most effective in their antiproliferative activities. They both share strong structural similarity with the difference being the functional group on carbon 4' where genistein has an hydroxy (-OH) group, and biochanin A a methoxy group ($-OCH_3$) (Figure 3-18; Figure 1-4). Daidzein does have a similar structure, but it lacks the hydroxy group on carbon 5 (found in both genistein and biochanin) (Figure 3-18; Figure 1-4). In contrast, daidzein has a hydrogen on carbon 5, thus missing the hydroxy functional group that both genistein and biochanin have on this carbon. Coumestrol is not an isoflavone, but a coumestan, which belongs to the isoflavonoid family. Coumestrol, therefore, has the most dissimilar structure from the other isoflavonoids (Figure 1-4), and like daidzein, it is less potent than genistein and biochanin A. It appears, therefore, that the hydroxy group on carbon 5 of the isoflavone structure is necessary, and so are the functional groups (hydroxy or methoxy) attached to carbon 4', in order for these compounds to exhibit potent antiproliferative properties in LNCaP and PC-3 cells.

The data presented here strongly show the antiproliferative effects of the isoflavones genistein and biochanin in both human prostate cancer cell lines, which

parallel the effects on other cancer cells as described in the literature. The present study sheds further light on the importance of the chemical structure of the isoflavones, and on the different mechanisms of genistein, and its precursor biochanin. They both have differential effect on the cell cycle phase distribution, and on some key cell cycle regulatory proteins in LNCa P cells (Figure 3-19; Table 3-2), and in PC-3 cells (Figure 3-20; Table 3-2). The two human prostate cancer cell lines, LNCaP and PC-3, have divergent reponses to the isoflavones. The IC_{50} values are generally 1 to 2-fold higher with PC-3 cells (Table 3-1). The latter are resistant to induction of apoptosis, whereas LNCaP cells readily undergo this type of programmed cell death with increasing concentrations of the isoflavones.

One key difference between these two cell lines is their p53 status. Many anticancer drugs are dependent on intact p53 suppressor gene function for their activity (Weinstein *et al.*, 1997). Among these anticancer drugs are 14 topoisomerase II inhibitors, including adriamcyn, bisantrene and VP-16 (Gupta *et al.*, 1995; Weinstein *et al.*, 1997). These topoisomerase inhibitors have been shown to be more potent in p53 wild-type cells than in mutant ones (Gupta *et al.*, 1995).

Cell culture studies are invaluable to assess the anticancer properties of these compounds in vitro. To map out, however, the mechanisms of action of drugs, the effects of these compounds need to be tested in an environment that is as close to a prostate tumor.

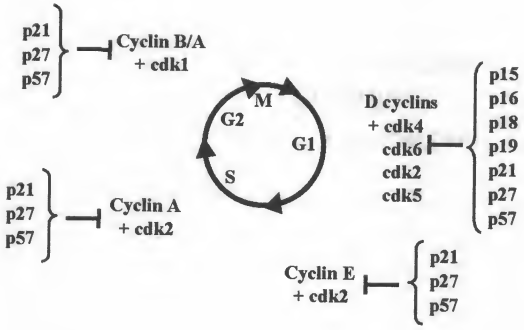
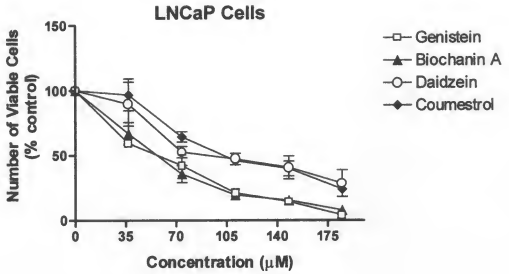


FIGURE 3-1. Cell cycle regulation. (Adapted from Lodish et al., 1996)

(A)



(B)

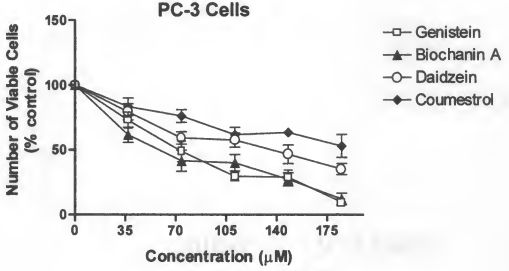


FIGURE 3-2. Dose-dependent effect of isoflavonoids on cell viability. Prostate cancer cells, LNCaP (A) or PC-3 (B), were treated for 48 hours with isoflavonoids at various concentrations or 0.1% DMSO (control). The number of viable cells was determined by trypan blue exclusion assay. Results from three experiments are expressed as mean ± standard error.

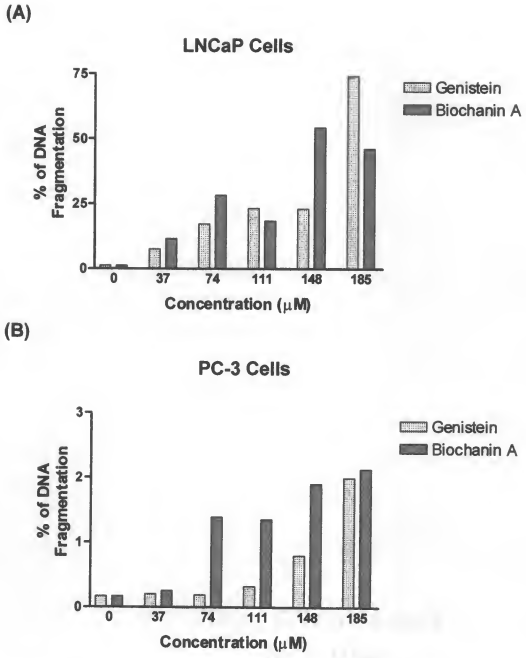


FIGURE 3-3. Dose-dependent induction of DNA fragmentation by the isoflavonoids. Prostate cancer cells, LNCaP (A) or PC-3 (B), were treated for 48 hours with isoflavonoids at various concentrations or 0.1% DMSO (control). The number of apoptotic cells was determined by the level of DNA fragmentation in the cells using a TUNEL assay. Data are from average of 2 experiments.

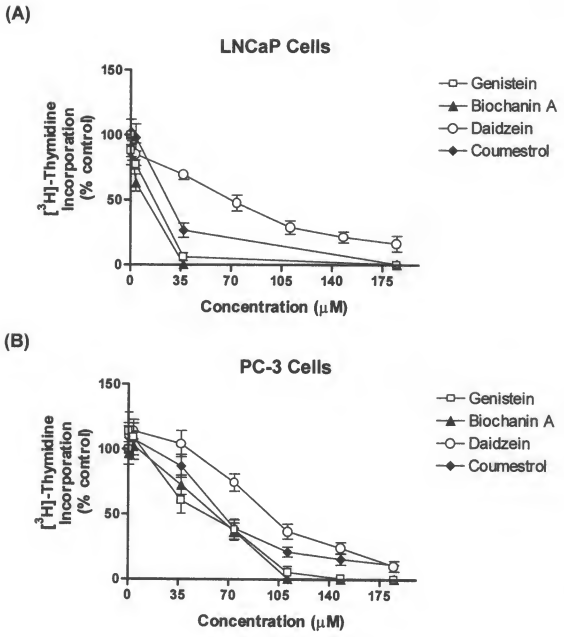


FIGURE 3-4. Dose-dependent inhibition of [^{3}H]-thymidine incorporation by the isoflavonoids. Prostate cancer cells, LNCaP (A) or PC-3 (B), were treated for 48 hours with isoflavonoids at various concentrations or 0.1% DMSO (control). DNA synthesis was measured by the level of ^{3}H-thymidine incorporation. Results from three experiments are expressed as mean ± standard error.

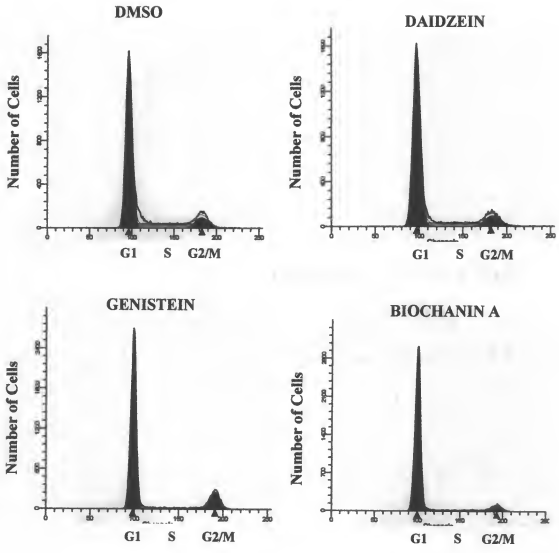


FIGURE 3-5. Effects on cell cycle phase distribution in LNCaP cells. LNCaP prostate cancer cells were treated for 48 hours with isoflavonoids at 37 μM or 0.1% DMSO (control). Cells were stained for their DNA content with propidium iodide. Analysis was performed by flow cytometry.

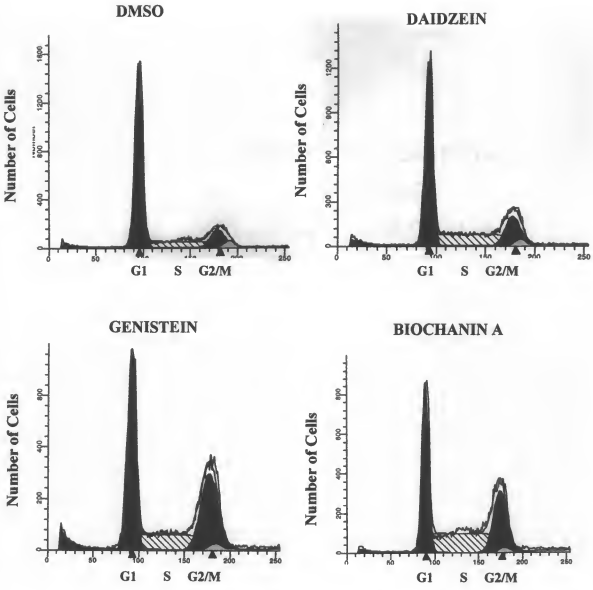


FIGURE 3-6. Effects on cell cycle phase distribution in PC-3 cells. PC-3 prostate cancer cells were treated for 48 hours with isoflavonoids at 111 μM or 0.1% DMSO (control). Cells were stained for their DNA content with propidium iodide. Analysis was performed by flow cytometry.

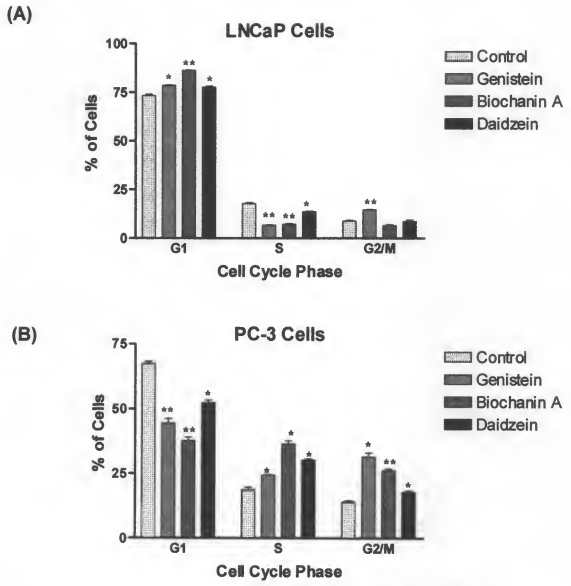


FIGURE 3-7. Summary of effects of isoflavonoids on cell cycle distribution. Prostate cancer cells, LNCaP (37 μM) (A) or PC-3 (111 μM) (B), were treated for 48 hours with isoflavonoids at their cytostatic doses or 0.1% DMSO (control). DNA content was determined by flow cytometry. Results from three experiments are expressed as mean ± standard error. * $p < 0.05$, ** $p < 0.01$.

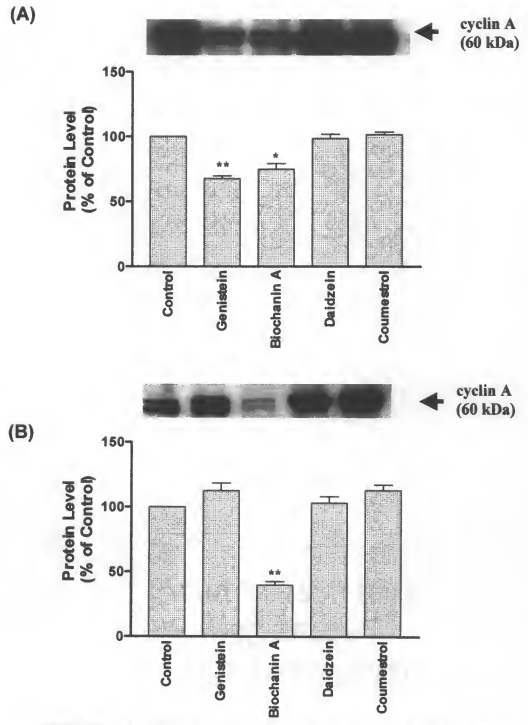


FIGURE 3-8. Effects on cyclin A protein level by the isoflavonoids. Data are from western immunoblot analysis of the effect on cyclin A in (A) LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. (B) PC-3 cells exposed to isoflavonoids at 111 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. * $p < 0.05$, ** $p < 0.01$ compared with control.

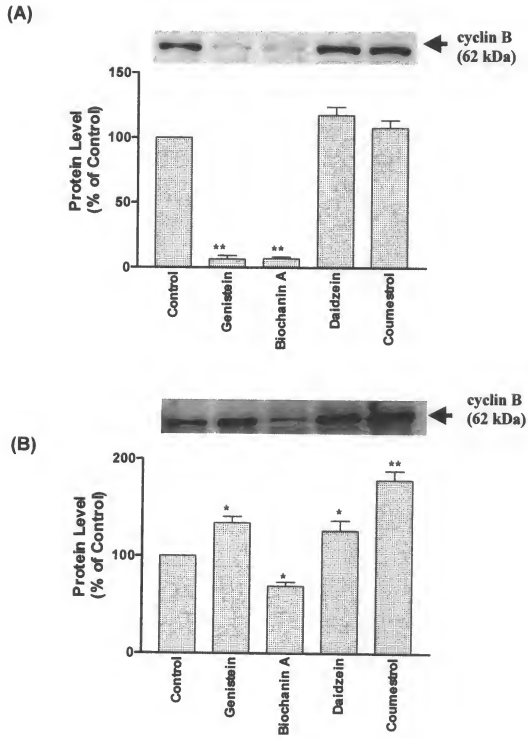


FIGURE 3-9. Effects on cyclin B protein level by the isoflavonoids. Data are from western immunoblot analysis of the effect on cyclin B in (A) LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. (B) PC-3 cells exposed to isoflavonoids at 111 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. * $p < 0.05$, ** $p < 0.01$ compared with control.

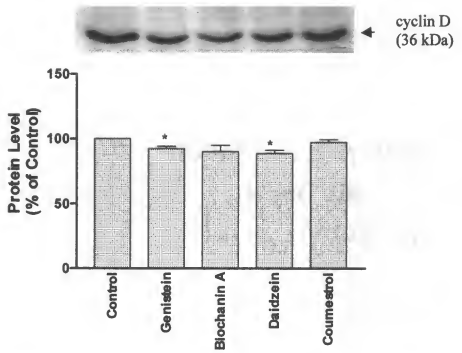


FIGURE 3-10. Effects on cyclin D protein in LNCaP cells. Data are from western immunoblot analysis of the effect on cyclin D in LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. * $p < 0.05$ compared with control.

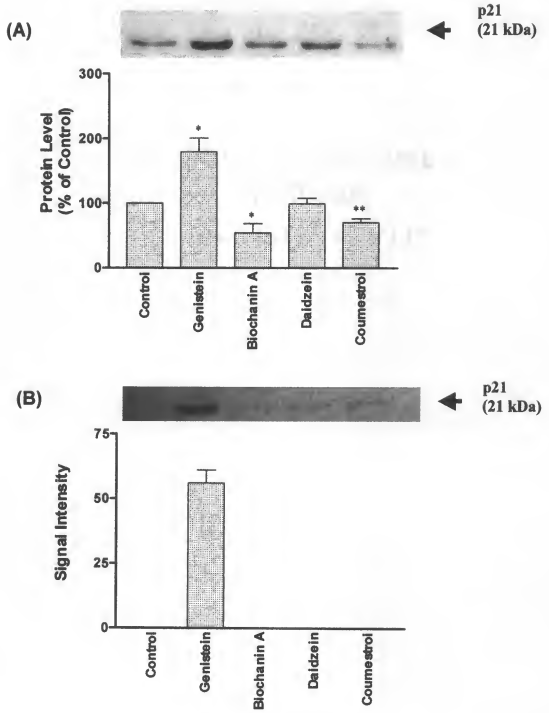


FIGURE 3-11. Effects on p21 protein level by the isoflavonoids. Data are from western immunoblot analysis of the effect on p21 in (A) LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. (B) PC-3 cells exposed to isoflavonoids at 111 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. * $p < 0.05$, ** $p < 0.01$ compared with control.

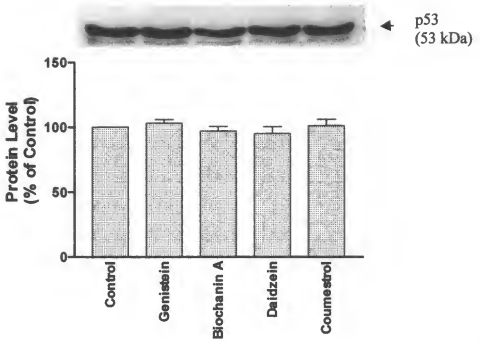


FIGURE 3-12. Effects on p53 protein level in LNCaP cells. Data are from western immunoblot analysis of the effect on p53 in LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error.

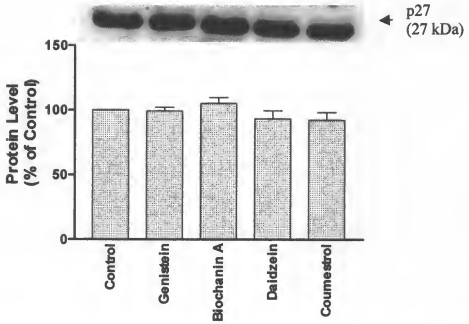


FIGURE 3-13. Effects on p27 protein level in LNCaP cells. Data are from western immunoblot analysis of the effect on p27 in LNCaP cells exposed to isoflavonoids at 37 μM or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error.

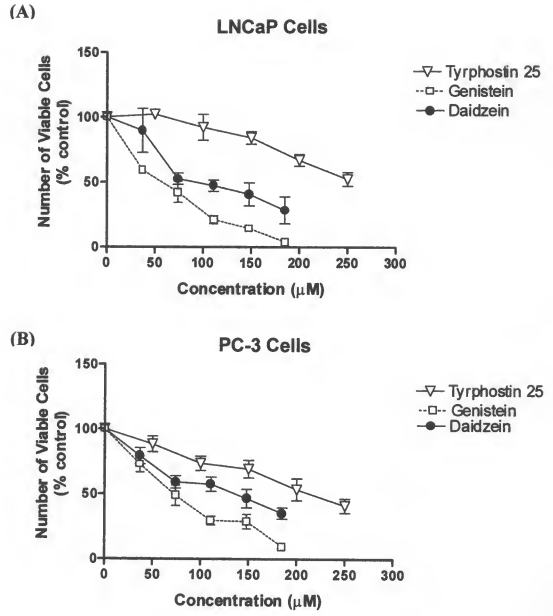


FIGURE 3-14. Effects of tyrphostin 25 on cell viability. Prostate cancer cells, (A) LNCaP or (B) PC-3, were treated for 48 hours with tyrphostin, genistein, daidzein at various concentrations or 0.1% DMSO (control). The number of viable cells was measured by trypan blue assay. Results from three experiments are expressed as mean ± standard error.

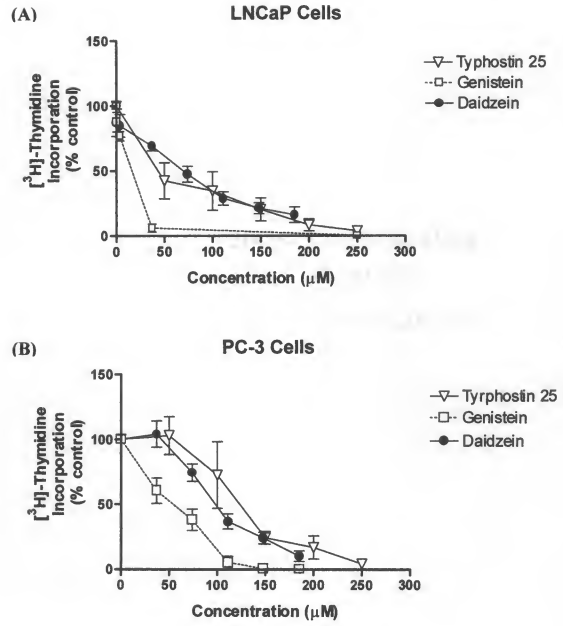


FIGURE 3-15. Dose-dependent inhibition of ^{3}H-thymidine incorporation by tyrphostin 25. Prostate cancer cells, (A) LNCaP or (B) PC-3, were treated for 48 hours with tyrphostin, genistein, daidzein at various concentrations or 0.1% DMSO (control). DNA synthesis was measured by the level of ^{3}H-thymidine incorporation. Results from three experiments are expressed as mean ± standard error.

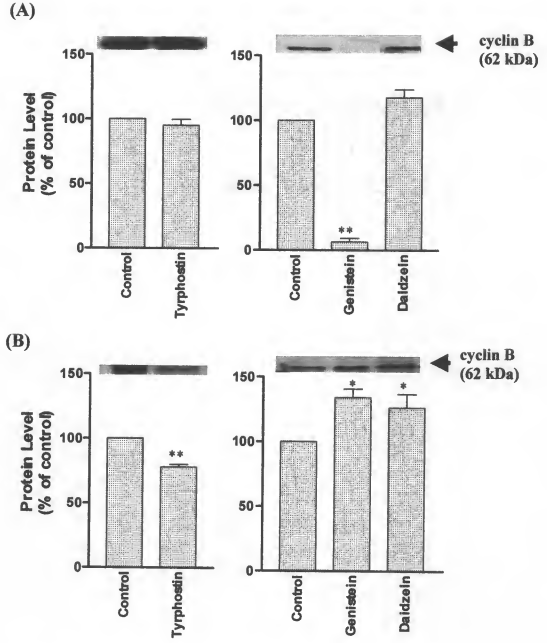


FIGURE 3-16. Effects on cyclin B protein level by tyrphostin 25. Data are from western immunoblot analysis of the effect on cyclin B in (A) LNCaP cells or (B) PC-3 cells exposed to tyrphostin at 250 μM, genistein, biochanin at 37 μM in LNCaP, at 111 μM in PC-3, or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. * $p < 0.05$, ** $p < 0.01$.

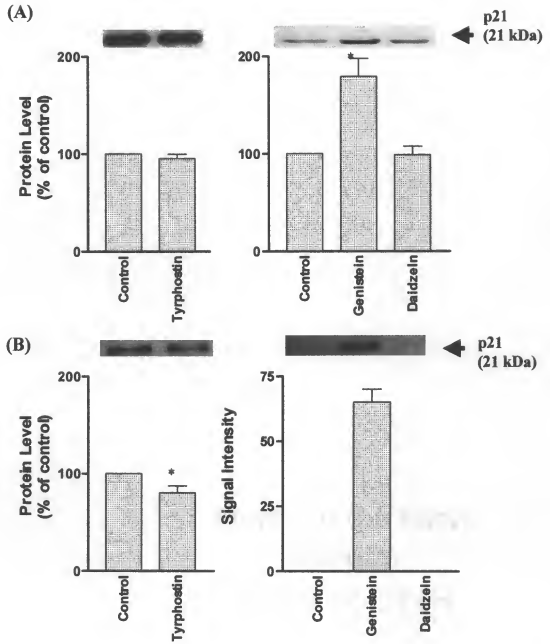


FIGURE 3-17. Effects on p21 protein level by tyrphostin 25. Data are from western immunoblot analysis of the effect on p21 in (A) LNCaP cells or (B) PC-3 cells exposed to tyrphostin at 250 μM, genistein, biochanin at 37 μM in LNCaP, at 111 μM in PC-3, or DMSO for 48 hours. Results from 3 experiments are shown as mean ± standard error. Note that p21 antibodies from 2 different sources were used (for tyrphostin, Transduction Lab, and for the isoflavones, Santa Cruz). * $p< 0.05$.

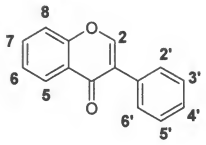


FIGURE 3-18. Conventional numbering schemes of the isoflavone chemical structure.

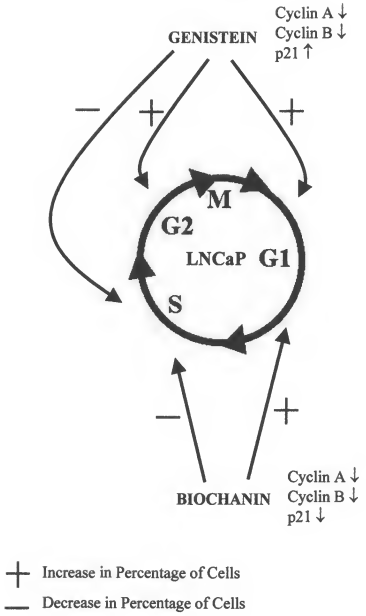


FIGURE 3-19. Summary of effects of isoflavonoids on LNCaP cell cycle.

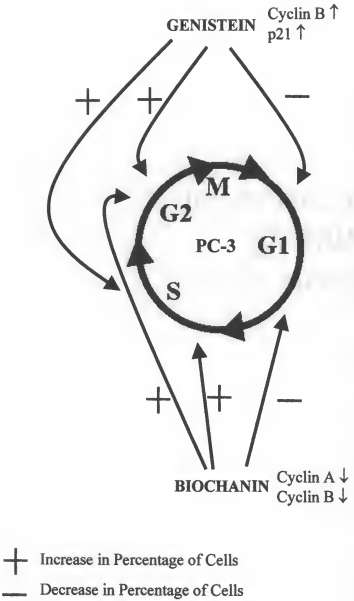


FIGURE 3-20. Summary of effects of isoflavonoids on PC-3 cell cycle.

Table 3-1. Summary of IC_{50} values for the effects on cell proliferation.

	IC_{50} values for effects on cell viability		IC_{50} values for effects on ^{3}H-thymidine incorporation	
	LNCaP	PC-3	LNCaP	PC-3
Genistein	63 μM	74 μM	17 μM	56 μM
Biochanin A	63 μM	63 μM	10 μM	63 μM
Daidzein	111 μM	150 μM	74 μM	100 μM
Coumestrol	111 μM	185 μM	33 μM	67 μM
Tyrphostin 25	250 μM	215 μM	47 μM	125 μM

Table 3-2. Summary of effects on cell cycle

	Cycle Arrest		Cyclin A		Cyclin B		Cyclin D		p21		p53		p27	
	LNP	PC3*	LNP	PC3	LNP	PC3	LNP	PC3	LNP	PC3	LNP	PC3	LNP	PC3
Genistein	G2/M	G2/M	↓	-	↓	↑	↓	na	↑	↑	-	na	-	na
Biochanin	G1	G2/M	↓	↓	↓	↓	-	na	↓	-	-	na	-	na
Daidzein	-	G2/M	-	-	-	↑	↓	na	-	-	-	na	-	na
Coumestrol	na	na	-	-	-	↑	-	na	↓	-	-	na	-	na
Tyrphostin	na	na	na	na	-	↓	na	na	↓	-	na	na	na	na

Note: LNP = LNCaP cells, PC3 = PC-3 cells, - = not significantly changed,
na = not assessed. * Genistein, biochanin and daidzein increased S phase

CHAPTER 4
IN VIVO EFFECTS

Introduction

In vitro studies are invaluable in the investigation of antiproliferative effects, and in the understanding of the mechanisms of action of the isoflavonoids on cancer cells. At the same time, there are clear limitations to cell culture data. In vitro systems have the obvious disadvantage that the cancer cells are cultured in an artificial environment, and the influence of the metabolism by the host as well as pharmacokinetic properties of the chemicals are lost (Mattern *et al.*, 1988). In vivo models are, therefore, essential for the study of anticancer activities of any potential agents, including the phytochemicals (Zhou *et al.*, 1999; Aronson *et al.*, 1999), on a prostate tumor in a more relevant biological environment (van Weerden and Romijn, 2000; Steel *et al.*, 1983). The effects of the isoflavonoids need to be assessed in a setting that can mimick as much as possible the biological milieu of a prostate tumor. Important physiological processes of such an environment include three-dimensional structure, angiogenesis (presence or lack of circulating activating/inhibiting factors), and stromal interactions (stroma-derived activating/inhibiting factors) (van Weerden and Romijn, 2000). These processes may influence tumor development, tumor growth, and tumor responsiveness to antiproliferative chemicals. There is, however, a lack of representative in vivo models for human prostate cancer. Animal models for prostatic cancer include the TRAMP transgenic model (Gingrich *et al.*, 1996), and the N-methyl-N-nitrosourea (MNU)

carcinogenesis model (Bosland, 1992; Bosland, 1999), but in general few are available since other mammal species, in contrast to men, do not, or only rather poorly, develop prostatic cancer spontaneously upon aging (Bosland, 1992). The alternative is to implant prostate cancer cells in an animal, and then the sensitivity of the resulting tumor to cytostatic or cytotoxic agents can be tested and determined (Mattern *et al.*, 1988). The use of mouse xenograft models has been extensive in prostate cancer research and is very helpful in studying the effects of the isoflavonoids in vivo (Bosland, 1999).

Human Tumor Xenograft Model

The Host Model

The introduction of the athymic mutant nude mouse as a host for heterotransplantation of human cancer tissue opened a new era for the xenograft models. These animals, which result from the inheritance of a recessive mutation, are virtually hairless, and exhibit thymus aplasia (Flanagan, 1966; Pantelouris, 1968). As a consequence of the absence of a functional thymus, the mouse mutant nude has a deficient cell-mediated immune response, with absence of T lymphocytes (Hanna, 1980). Although humoral antibody formation is only slightly impaired and the activity of natural killer (NK) cells is actually increased in these immunosuppressed animals, successful transplantation of human malignant tissue resulted in tumors without additional immunosuppression (Cobb and Mitchley, 1974; Povlsen and Rygaard, 1971; Rygaard and Povlsen, 1969).

Anchorage-Mediated Tumor Growth

Natural basement membrane. Basement membranes are thin but continuous sheets that separate epithelium from stroma and surrounding nerves, muscle fibers, smooth muscle cells, and fat cells (Kefalides, 1973; Vracko, 1974). Basement membranes contain primarily type IV collagen (Kefalides, 1973), the glycoproteins laminin (Timpl *et al.*, 1979), entactin (Carlin *et al.*, 1981), nidogen (Timpl *et al.*, 1983), and heparan sulfate proteoglycan (Kanwar and Farquhar, 1979). These extracellular matrix components are codistributed both within the lamina densa and within its extensions across the lamina lucida (Hayman *et al.*, 1982; Leivo *et al.*, 1982). Their codistribution suggests that the formation of basement membrane occurs through their interactions. Type IV collagen molecules form intermolecular disulfide bonds and associate in a continuous network (Eyre, 1980). Laminin binds through its short chains to native type IV collagen and through a domain in its long chain to the heparan sulfate proteoglycan (Rao *et al.*, 1982; Woodley *et al.*, 1983). These macromolecules form the matrix structures that contribute to the physical characteristics of tissues as well as provide unique substrates, attachment support, and growth signals for the resident cells.

Reconstituted basement membrane matrix (Matrigel®). Many cancer cells (including LNCaP cells), when injected subcutaneously in nude mice, do not develop into a tumor (Fridman *et al.*, 1990). However, coinjection of cancer cells with a reconstituted basement membrane matrix, from the extracellular matrix of Schwann cells, promotes the development of a tumor (Lim *et al.*, 1993; Fridman *et al.*, 1991). As described above, the major components of the reconstituted structures include laminin, type IV collagen, heparan sulfate proteoglycan, entactin, and nidogen. Under physiological conditions,

these materials polymerize in constant proportions on reconstitution (Kleinman *et al.*, 1986). At 37 °C, these components form a gel with lamellar structures resembling in width those in basement membranes; however, at 4 °C, these components form a liquid (Kleinman *et al.*, 1986).

Biological interactions between Matrigel® and cancer cells. When tumor cells are coinjected with Matrigel®, they form a cluster in a basement membrane-like gel, which appears to sustain their growth. The biological interactions of the cancer cells with the Matrigel® are primarily with laminin (Figure 4-1) (Albini *et al.*, 1987; Kleinman *et al.*, 1987), one of its main components. Although tumor cells interact with multiple proteins in the Matrigel®, they derive most of their growth promoting factors from laminin. This protein promotes tumor cell adhesion, migration, growth, collagenase IV activity (invasiveness) (Barsky *et al.*, 1984; Liotta *et al.*, 1986; Terranova *et al.*, 1984).

Growth Characteristics

Xenograft tumors tend to grow as well-circumscribed nodules at the site of inoculation without infiltration into the surrounding connective tissue (Sharkey and Fogh, 1979). The vascular system and the supporting stromal elements originate from the host, whereas the tumor parenchyma is of human origin. Tumor nutrition is also of host origin. In most cases, the tumors initially exhibit an exponential growth, followed by a slowing of the growth rate. Sometimes, however, tumor growth is irregular (Rofstad *et al.*, 1982). Xenograft tumor volume doublings are considerably faster than in human tumors, usually 2 to 8 times (Steel and Peckham, 1980).

Experimental Model

Experiment Model I

This model evaluated the ability of biochanin A to inhibit the establishment of a tumor. On day 0, viable 5 x 10^6 LNCaP cells in 300 µl of serum-free medium were coinjected with matrigel subcutaneously in both right and left flanks, for a total of 10 x 10^6 LNCaP cells per animal (Figure 4-2). Mice were randomly assigned to receive daily intraperitoneal injections of either 400 µg biochanin in 100 µl DMSO or vehicle alone for Days 1-10 (mice were not treated with genistein in this experimental model). Body weight, tumor incidence were measured at 3, 4, 5 and 6 weeks post-implantation. Tumor volume was measured with calipers weekly until the mice were sacrificed at 8 weeks post-implantation. Length (L), width (W) and height (H) of the tumors were measured and volume (V) is calculated where V = L x W x H.

At sacrifice, blood samples were collected from the mice for analysis. Radio-immuno assays for testosterone were performed on 40 serum samples by Ocala Veterinary Endrocrine Laboratories (Ocala, FL). Tumors were extracted and stored for further immunohistochemical analysis.

Experiment Model II

This model examined effects of biochanin A and genistein on the growth of established tumor. Mice implanted (in the right flank only) with LNCaP cells as described for Experiment I, were randomly assigned to treatment groups when tumors measured 0.2 cm^3. Mice were then injected with biochanin, genistein or vehicle alone daily for 10 days (Figure 4-3). Tumor measurements were recorded bi-weekly and the

mice sacrificed 3 weeks from the start of treatment. Body weight and tumor volumes were recorded as in model I. Similar procedures, as in model I, were performed at sacrifice. In addition, tumors from sacrificed animals were analyzed by immunohistochemistry, see Chapter 2 for methods.

Data Analysis

The evaluation of treatment effects in xenografts has relied largely on changes in tumor volume (Shorthouse *et al.*, 1980). For model I, differences in incidence and tumor volume between treatment groups were determined using a two-sample t-test analysis. For Model II, relative tumor volume (RV) (Mattern *et al.*, 1988) was calculated for each mouse where $RV = V_x/V_0$, V_x is the actual volume measured from day x, and V_0 is the starting volume at the start of the treatment. Relative volume is important since the size of the tumors at the start of the treatments can vary. Two-way ANOVA analysis was performed to assess the significance of the effects of the isoflavonoids treatment, and time exposure in the xenograft nude mice. Statistical analysis was using Prism 2.01 software.

Results

Data from Experimental Model I

Effects on tumor growth. Mean tumor volume in biochanin-treated mice was smaller compared to controls at 3 and 6 weeks ($p < 0.01$) post-implantation of LNCaP xenografts (Figure 4-4; Figure 4-5). However, by 9 weeks, the variation in tumor size was greater and there was no significant difference between groups. Tumor incidence in biochanin-treated mice at 3 weeks was significantly lower, 54% compared to 89% for

control mice ($p < 0.05$), whereas at 6 wks, tumor incidence was the same for the two groups (Figure 4-6).

Data from Experimental Model II

Effects on Tumor Growth. In this model, LNCaP cells were coimplanted with Matrigel® in the nude mice. When tumor size had reached at least 0.2 cc, they were randomly assigned to groups, which received either genistein, biochanin or DMSO for 10 days. The experiment terminated 14 days from the last day of treatment. In the first experiment which compared only DMSO and biochanin, ANOVA analysis indicated there was a significant main effect of biochanin on the prostate tumor growth ($p < 0.0001$) (Figure 4-7A). At the end of this first experiment, the mean tumor volume of biochanin-treated mice was 42% lower than the controls. In a second experiment, with identical design as the first one, except a genistein group was added. ANOVA analysis showed there was significant main effect with biochanin ($p = 0.0267$) and genistein treatment ($p = 0.0431$) (Figure 4-7B). At the end of the second experiment, the mean tumor volumes of genistein-treated and biochanin-treated mice were 42% and 37% lower than controls, respectively.

Immunohistochemical Analysis. Tumors were collected at time of sacrifice and processed for immunohistochemical analysis. H&E staining first was used to evaluate the number of cells with mitotic figures. In the first experiment, at 3 weeks, the level of cells undergoing mitosis was significantly changed in tumors of mice treated with biochanin with an average of 2.9 mitotic cells per field, compared to 4.1 in controls ($p = 0.044$). In the second experiment, tumors from biochanin-treated mice had comparable average of mitotic cells to controls per field, 4.5 vs 4.8 in tumors of DMSO-treated

animals ($p = 0.801$). In the same experiment, however, genistein increased the number of mitotic cells at 3 weeks (14 days after the end of treatment) with an average of mitotic cells of 6.6 per field (Figure 4-8), compared to 4.8 in controls ($p = 0.076$).

Tumor tissues were also evaluated for angiogenesis markers by immunohistochemistry staining for CD34 antigens. In the first experiment, the level of CD34 staining was comparable, with no significant changes, between tumors of biochanin and DMSO-treated animals, 8.6 compared to 9.4 vessels per field in controls ($p = 0.73$) (Figure 4-9). A similar observation was made in the second experiment between tumors of biochanin-treated and DMSO-treated mice, 22.1 vs 19.87 microvessels per field ($p = 0.70$). This suggests that formation of new blood vessels was not affected by the isoflavone treatment, or at least the difference was observed at 3 weeks. Tumors from mice receiving genistein were not evaluated for CD34 staining.

Discussion

Animal models are crucial in preclinical efficacy testing for chemoprevention agents. These model systems allow not only evaluation of the responsiveness to a potential therapeutic agent, but also to elucidate modes of action through analysis of the tumors from treated and non-treated host animals. Using the appropriate experimental models, certain biological questions can only be answered in an in vivo setting. The study from experimental model I showed that biochanin-treated mice had some protection against tumor growth as compared to DMSO-treated mice. This agrees with reports in the literature of the inhibition of LNCaP tumor growth in mice by soy isoflavone concentrates (Zhou *et al.*, 1999), and by soy protein extracts (Aronson *et al.*, 1999; Bylund *et al.*, 2000). Similar inhibitory effects have been reported with biochanin

in other tumor models, including mouse lung tumor induced by benzo(a)pyrene (Lee *et al.*, 1991). Furthermore, biochanin has been shown to prevent (MNU)-induced mammary tumors in rats (Gotoh *et al.*, 1998). The present study is the first to investigate the effects of individual isoflavones on the establishment of LNCaP tumor xenografts in an animal model. Treatment for ten days, which started the day after implantation of LNCaP cells, decreased the incidence of tumors, and delayed growth of the tumors. However, these growth inhibitory effects tapered off with time. Future experiments should include monitoring the mice every day for the exact time of tumor appearance. This information is important since it would tell whether the isoflavones affect the time of appearance of the tumors in the nude mice.

The fact that biochanin-treated mice had similar tumor incidence and tumor volume at 6 and 9 weeks in model I raises the question of whether the tumors have become more aggressive after treatment, or whether a resistant population of cells has emerged. Continuous treatment may be needed to sustain the antitumorigenic effects in the mice, and to test the possibiltiy of a group of resitant prostate cancer cells. This experimental model addressed the issue of chemoprevention more directly since the mice were exposed to the isoflavone before the tumors were established (Swan and Ford, 1997). This model has relevance for men who are continuously exposed to a soy diet early on in life.

In the experimental model II, the present study addresses a different question, which is whether the individual phytochemicals can affect the growth of an established prostate tumor. This study is the first to address this issue. It was found that genistein and biochanin significantly decreased the growth rate of established tumors in the

xenograft nude mice over a 3 week period. In this regard, the antitumorigenic effects of genistein have previously been investigated in rat colon cancer models, where it was reported that this isoflavone can inhibit aberrant colonic crypt formation induced by carcinogens (Pereira *et al.*, 1994; Thiagarajan *et al.*, 1998). Genistein has been shown to lower the incidence, multiplicity and tumor size in the DMBA-initiated, phorbol ester-promoted skin cancer in mice (Wei *et al.*, 1995). In addition, genistein is known to suppress mammary cancer in rats (Lamartiniere *et al.*, 1998). In the present study, the model II addresses the issue of using the isoflavonoids as chemotherapeutic agents rather than chemopreventive ones (Mattern *et al.*, 1988). This model has relevance for men who may wish to adopt a soy diet if they are diagnosed with prostate cancer, or if they are at risk for this type of cancer. Since the mice were sacrificed at 3 weeks from the initiation of the treatment, little can be said about the full effects of these phytochemicals on growth retardation of tumors over longer periods.

Isoflavonoids, such as genistein, has been shown to suppress angiogenesis by inhibiting the proliferation of endothelial cells in vitro (Fotsis *et al.*, 1993, 1995, 1997, 1998). In addition, preliminary results from Dr. Siemann's laboratory found genistein, but not biochanin, to inhibit the proliferation of mouse heart (MHE) endothelial cells. In the present study, however, immunohistochemical analysis revealed no significant differences in the level of CD34 staining between tumors of DMSO-treated and biochanin-treated mice at 3 weeks. CD34 is a marker for endothelial cells, and indirectly for blood vessels (Miettinen *et al.*, 1994). The inhibitory effects of the phytochemicals may be due to events other than the inhibition of angiogenesis, such as direct antiproliferative effects on tumor cells. Alternatively, the effects of the isoflavones on

angiogenesis may have occurred earlier than 3 weeks, which could explain the growth retardation seen at 3 weeks. This finding differs to what was reported in mice transplanted with human LNCaP xenografts and treated with soy isoflavone concentrate, where angiogenesis was shown to be inhibited as measured by factor VIII staining (Zhou *et al.*, 1999). Furthermore, in the present study, the number of cells with mitotic figures did not significantly change with biochanin treatment, but it was significantly increased with genistein treatment at 3 weeks. This higher level of mitotic cells in tumors of genistein-treated mice may be the result of a rebound effect in the tumor growth after the treatment has stopped, an effect which clearly merits further study.

The dose of the isoflavonoids used in this study has been shown by other groups to be a non-toxic dose in the nude mouse, based on body weight and general health. However, it may not be the maximum-tolerated dose (MTD) or the minimum-effective dose (MED). Therefore, different responses may be obtained at other doses. Furthermore, the in vitro studies described in chapter 3 showed that genistein and biochanin A share similar and different modes of action, so they may be effectively used in combination at lower doses to test for synergistic or additive effects in vivo.

There are a number of differences that exist between tumors in humans and tumors that are xenografted in nude mice. Tumor cell growth kinetics and growth rate are much higher in animals. This is especially true for prostate tumors, which grow slowly in humans and may take years to become clinically detectable. In addition, human LNCaP xenografts maintained subcutaneously, in the nude mice, grow as well-circumscribed nodules at the site of inoculation without local invasion and no metastasis.

The pharmacodynamics and pharmacokinetics of the isoflavonoids in the mice are likely different in humans. In humans, biochanin after ingestion is rapidly and efficiently demethylated, resulting in high plasma concentrations of genistein (Setchell *et al.*, 2001). The apparent volume of distribution of genistein and biochanin in humans suggests extensive tissue distribution (Setchell *et al.*, 2001). This would imply that genistein and biochanin would have similar pharmacodynamics in humans, but little is known about the bioavailability of the isoflavones in nude mice. In addition, the isoflavonoids have been shown to be metabolized by intestinal bacteria (Axelson *et al.*, 1982, 1984). The intraperitoneal route of administration used in the present study precludes intestinal metabolism. Nevertheless, even with these limitations, the xenograft models add a valuable in vivo profile to the potential chemopreventive effects of the soy chemicals.

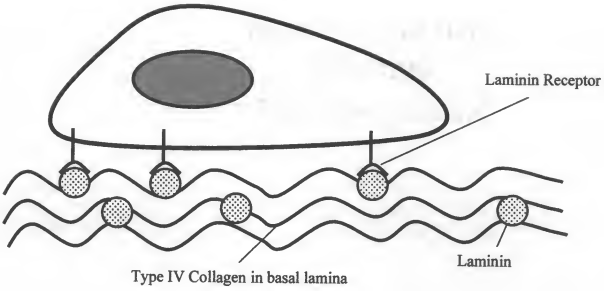


FIGURE 4-1. Interactions of cells with laminin in the basement membrane.

In Vivo Experimental Model I

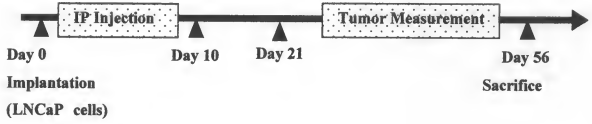


FIGURE 4-2. In vivo experimental model I. Nude mice were injected with 100 µl of DMSO alone or containing 400 µg biochanin A for 10 days, starting at day 1 after implantation. Body weight and tumor volumes were measured twice a week from day 21 to day 56.

In Vivo Experimental Model II

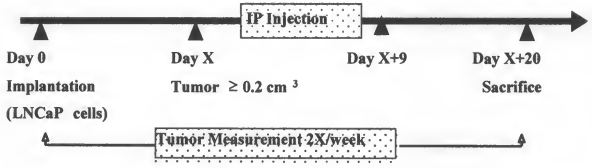


FIGURE 4-3. In vivo experimental model II. Nude mice were injected with 100 μl of DMSO alone or containing 400 μg of either genistein or biochanin A for 10 days, starting at day X, when the tumor size reached 0.2 cc. Body weight and tumor volumes were measured twice a week.

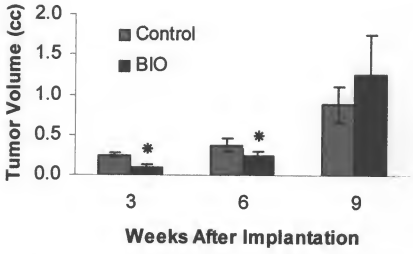


FIGURE 4-4. Effects on tumor growth from design I. Growth of LNCaP tumors in xenografts mice treated with Biochanin A for 10 days immediately after implantation, compared with controls (n=6-7 animals in each group). Tumor volume was significantly smaller in BIO treated-mice at 3 and 6 wks post-implantation. Data are shown as the mean ± standard error. $*p<0.01$

FIGURE 4-5. Representative xenograft nude mice. Biochanin-treated mice had smaller tumor size than control-treated mice.

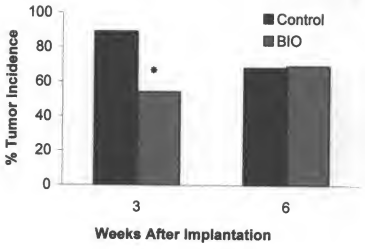
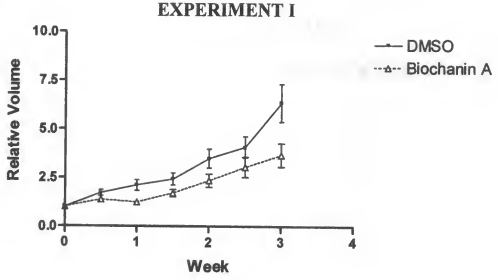


FIGURE 4-6. Tumor incidence from experiment design I. Post-implantation treatment with biochanin for 10 days reduced the tumor incidence in xenograft nude mice from 89% to 54% at 3 weeks, but not at 6 weeks post-implantation. $^{*}p<0.05$ (probability Z-test).

A)



B)

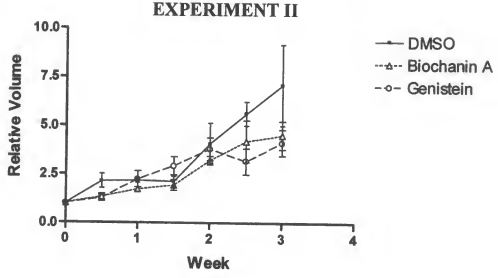
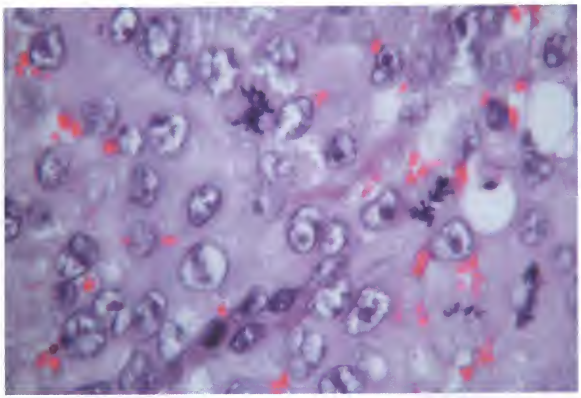


FIGURE 4-7. Effects on tumor growth from design II. (A) In experiment I, biochanin A (n = 7) lowered the growth curve of established tumors (≥ 0.2 cc) as compared to vehicle-treated mice (n = 7). (B) In experiment II, Biochanin A (n = 7), and genistein lowered the growth curve of established tumors (≥ 0.2 cc) as compared to vehicle-treated mice (n = 7).

A)



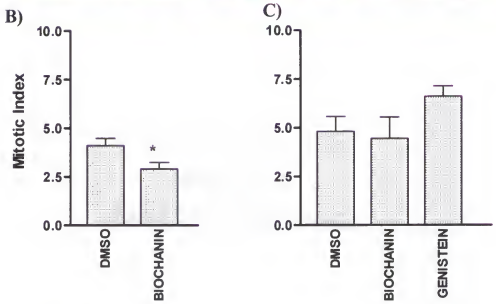


FIGURE 4-8. Hematoxylin and eosin staining of tumor tissues from design II. A) Representative tumor tissue with mitotic cells. B) Level of mitotic cells in tumors of DMSO-treated and biochanin-treated mice from Experiment I. C) . Level of mitotic cells in tumors of DMSO-treated and biochanin-treated mice from Experiment II. Data represent mean ± standard error (n = 7 per groups. * $p < 0.05$

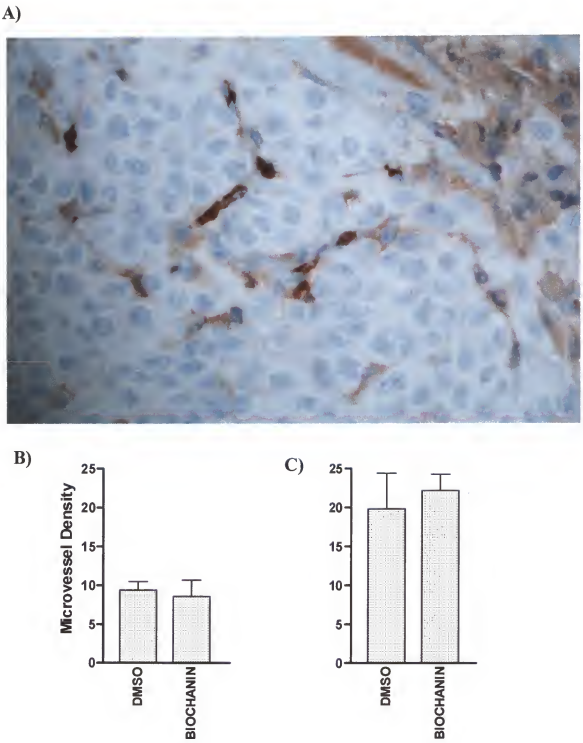


FIGURE 4-9. Microvessel density in tumor tissues from design II. A) Representative tumor tissue with CD34 staining. B) Microvesel density in tumors of DMSO-treated and biochanin-treated mice from Experiment I. C) . Microvesel density in tumors of DMSO-treated and biochanin-treated mice from Experiment II. Data represent mean ± standard error (n = 7 per groups).

CHAPTER 5
EFFECTS ON GENE EXPRESSION

Introduction

"Exploration means looking around, observing, describing, and mapping undiscovered territory, not testing theories or models" (Brown and Botstein, 1999). The goal is to discover things we neither knew nor expected, and to see relationships and connections among the elements, whether previously suspected or not. "It follows that this process is not driven by hypotheses and should be as model-independent as possible" (Brown and Botstein, 1999). Most current drugs have been developed by identifying biochemical pathway involved in a pathophysiological process (Debouck and Goodfellow, 1999). For example, once a relevant enzymatic activity, ideally the rate-limiting step in a given pathway is characterized, the purified enzyme may then be screened against a series of structurally diverse molecules (Kuntz, 1992; Lednicer, 1987). These agents are usually selected based on what is known about the mode of action and structure of the targeted enzyme. Finally, the leading molecule (based on specificity) becomes the chosen drug. A similar approach is used to identify receptors and their use as drug targets (Luyten and Leysen, 1993; McLean, 1989). The biochemistry-based approach is an ideal model for developing a number of effective drugs for a variety of clinical conditions. However, the advent of DNA microarray technology has revitalized and transformed exploration in biological research, including drug discovery. Such technology can be used to measure variations in gene expression patterns in a cell in

response to drug treatments (Cunningham, 2000; Michelson and Joho, 2000; Sausville and Johnson, 2000).

The use of DNA microarray analysis can yield an added profile of a potential pharmaceutical compounds by revealing the effects on gene expression, thus identifying drug targets (Marton *et al.*, 1998). There exists a tight connection between the expression pattern of a gene and its function. Furthermore, there is a correlation between expression level of a gene and its activity, and the need of the cell for this gene at the time point observed (Clinton *et al.*, 2000; Stuart *et al.*, 2001). The gene expression pattern produced by a drug can become its fingerprint under the given treatment conditions (Scherf *et al.*, 2000). Therefore, microarrays are potentially powerful tools to investigate the mechanisms of action of a drug, to test one's hypothesis in a more global picture, and to even generate new hypotheses.

The isoflavonoids genistein and biochanin A exert a variety of effects on the LNCaP and PC-3 cancer cells as described in the previous chapters. To obtain an overall picture of the gene targets of these phytochemicals, the gene-by-gene investigation approach will not suffice. It is necessary to take "global views" of biological processes by simultaneous readouts of all components (Lander, 1999). DNA microarrays offer great possibilities for such investigation, and may help shed more light on mechanisms of action that were neither known or expected about these phytochemicals.

Principle of the Method of DNA Microarray Technology

DNA microarray technology is possible mainly because of the feature of the DNA duplex. Despite of its great structural complexity, a DNA molecule can reassemble with perfect fidelity from the separated strands (Southern *et al.*, 1999). The ability of nucleic

acids to hybridize (one of which is immobilized on a matrix) is very much exploited in molecular biology, including this new technology (Schena *et al.*, 1995). This method yields high sensitivity and specificity of detection as a consequence of exquisite natural selectivity between complementary strands of nucleic acids (Duggan *et al.*, 1999). The field has evolved from the discovery that labeled nucleic acid molecules could be used to interrogate nucleic acid molecules attached to a solid support (Southern, 1975). Historically, most applications of this approach have utilized individual labeled oligonucleotides or polynucleotides attached to a solid support. In Northern blotting, transcript abundance is determined by immobilizing RNA on membranes and then incubating with a radioactive labeled gene-specific target. If more than one RNA sample is immobilized on the same matrix, information is obtained about the quantity of a particular message present in each RNA pool (Alwine *et al.*, 1977; Taniguchi *et al.*, 2001).

DNA microarrays expand the possibilities of this method. In a given array experiment, several hundreds, even thousands of gene-specific polynucelotides derived from the 3' end of cDNA transcripts are individually immobilized on a single matrix. For membrane-based arrays, the matrix is usually nitrocellulose, or charged nylon (Cheung *et al.*, 1999; Duggan *et al.*, 1999). The making of an array starts with the selection of probes to be immobilized on the matrix. In most cases, these probes are selected directly from databases, including GenBank (Benson *et al.*, 1999), dbEST (Boguski *et al.*, 1993), UniGene (Schuler *et al.*, 1996), I.M.A.G.E. (Lennon *et al.*, 1996), or other databases of interest. cDNA arrays are then produced by spotting PCR products (0.6 - 2.4 kb) representing specific genes onto a matrix. Printing is achieved using a robot that spots a

sample of each sequence onto a number of matrices in a serial operation. In most instances, DNA is cross-linked to to the matrix by ultraviolet irradiation (Cheung *et al.*, 1999).

The microarray analysis is performed by probing a matrix, in parallel with another matrix, with radiolabeled reverse-transcribed cDNA representations of total RNA pools from cells or tissues. Comparisons between hybridizations,parallel or serial, allow the determination of the relative amount of these transcripts (those immobilized on the matrix) present in the pool by the level of radioactive signal emitted (Figure 5-1) (Duggan *et al.*, 1999). Computing and analysis are performed using commercially available software, which processes array images. The basic goal is to reduce an image of spots of varying intensities into a table with a measure of the intensity for each spot. Scanning and image processing may require human intervention to ensure that grids are properly aligned (Cheung *et al.*, 1999).

Human Prostate-Specific Microarray Membranes

For this project, a human prostate-specific microarray membrane (5 cm x 7 cm) was used, which contains cDNA sequences coding for 2,000 known genes, and 1500 expressed sequence tage (ESTS) (Figure 5-2). The sequences spotted on the membrane have been shown to be expressed in normal or malignant prostate tissue. Each spot on the membrane contains approximately 0.5 ng of insert DNA from a cDNA clone (approximately 1000 bases from the 3' end of the gene) obtained from Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) library (Lennon *et al.*, 1996). The insert has been denatured and UV cross-linked to a positively charged

membrane. All cDNA clones on the human prostate membrane array have been isolated, sequenced and verified to be correct by Research Genetics.

The membrane contains two fields numbered 1 and 2. Each field is divided into 8 grids labeled from right to left as A-H, with each grid being organized into 30 rows and 12 columns. The right-hand column in each grid contains control DNA in every other row for the first 12 rows, in every row for the next 12 rows and then agin in every other row for the final 6 rows. The second column from the right also contains control DNA in every other row for the first 6 rows. The spotting pattern of control sequences is used to orient the membrane. The remaining spots contain the partial sequences of genes whose expression pattern will be evaluated.

Methods for Analyzing Expression Data

Normalization and Relative Fold-Changes

To successfully carry out comparisons of expression data from measurements of serial or parallel hybridizations, normalization of the data is necessary. Even though all expressions are carried out under similar conditions, variations may arise from amount of applied probe, extent of target labeling, or the efficiency of signal emission from radiolabeled nucleotides. There are essentially two main strategies for normalizing the data (Duggan *et al.*, 1999). One is derived from the consideration of all of the genes in the matrix, and the other is based on considering a designated subset expected to be unchanging over most hybridizations. When using closely related samples, the transcript level of many genes will remain unchanged, and global normalization becomes useful. As samples become less similar, the fraction of genes revealing altered transcript levels increases, such that a better estimate of normalization is achieved by using a subset of

sequences that show a stable expression pattern across experiments and conditions. Identification of the gene expression profile of drug-treated cells is achieved by obtaining fold-changes in expression of genes relative to control-treated cells. Genes having fold-changes above a desired cut-off (e.g., 2-fold or greater up or down) are selected for further considerations (Ermolaeva *et al.*, 1998).

Data Mining

Obtaining fold-changes is relatively simple. Extracting the full meaning and implications of such a high-volume data set, however, is a great intellectual challenge. "Data Mining" is defined as "the exploration and analysis by automatic or semi-automatic means, of large quantities of data in order to discover meaningful patterns and rules." (Bassett, Jr. *et al.*, 1999). The ultimate objective is to convert expression data into a biologically meaningful context (Ouellette and Boguski, 1997). There are two general approaches to data mining, hypothesis testing and knowledge discovery. Hypothesis testing is a top-down approach where there is some understanding of the biological process happening in the cell under the conditions being investigated by microarray analysis. Thus, this approach will lead to predicted results and to a search of only interested genes. Knowledge discovery by exploratory data analysis is a bottom-up approach where the data can generate hypotheses (Brown and Botstein, 1999).

Cluster Analysis

Background. One of the most basic human abilities involves the grouping of similar objects to produce a classification. The idea of sorting similar things into categories is clearly a primitive one since classification, in the widest sense, is needed for the development of language which consists of words which help us recognize and

discuss the different types of events, objects, and people. As well as being a basic human conceptual activity, classification is also fundamental to most branches of science. In biology, for example, classification of organisms has been a preoccupation since the very first biological investigations (Everitt, 1993). Aristotle built up an elaborate system for classifying the species of animal kingdom those having red blood (corresponding roughly to our own vertebrates) and those lacking it (the invertebrates) (Everitt, 1993).

In general, a classification scheme may represent simply a convenient method for organizing a large set of data such that the retrieval of information may be made more efficiently (Kauffman, 1990). Describing patterns of similarity and differences among the objects under investigation by means of their class labels may provide a very convenient summary of data. Numerical techniques for devising classifications originated largely in biology and zoology in order to rid taxonomy of its traditionally subjective nature and to provide objective and stable classifications (Jain and Dubes, 1988). A number of names have been applied to these numerical methods depending largely on the area of application. Numerical taxonomy is generally used in biology (Sneath and Sokal, 1973). In other fields, it may be known as Q analysis, unsupervised pattern recognition, clumping and grouping. At the present time, the most common generic term is cluster analysis (Everitt, 1993).

Cluster. What is a cluster? The terms cluster, group and class are usually used in an essentially intuitive manner without any attempt at formal definition. It turns out that such a formal definition is not only difficult but may even be misplaced. It has been suggested that the ultimate criterion for evaluating the meaning of such terms is the value judgement of the user. If using a term such as "cluster" produces an answer of value to

the investigator, that is all that is required (Everitt, 1993). Cluster analysis is a powerful tool for exploring enormous data sets such as expression data from cDNA microarray technology. The basic problem about any body of data is to make it more easily and effectively understandable to our minds. Cluster analysis is therefore often supplemented by techniques for visualizing the data. It becomes easier to search for clusters in a data set by examining some relatively simple graphical displays. Well chosen graphical displays can enhance the understanding of the data and the ease for identifying patterns of expression (Eisen *et al.*, 1998).

Hierarchical Clustering. The idea of clustering genes on the basis of their expression patterns predates the microarray technology and has been applied to expression studies. For example, the identification of gene expression at various points in the cell cycle has been used to cluster groups of genes (Stein *et al.*, 1980). However, the advent of microarray technology now allows for clustering on a genomic scale. The first step toward grouping genes with similar patterns of expression is to adopt a mathematical description of similarity. For any series of measurements, the measure of similarity in the behavior of two genes can be obtained by the dot products of the two n-dimensional vectors representing a series of n measurements (Eisen *et al.*, 1998). The standard correlation coefficient (the dot product of two normalized vectors) conforms well to the intuitive biological notion of what it means for two genes to be "coexpressed" (Eisen *et al.*, 1998).

The gene similarity metric used in this project is a form of correlation coefficient. Let G_i equal the (variance-normalized) primary data for gene G in condition i. For any

two genes X and Y observed over a series of N conditions, a similarity score is computed as follows:

$$S(X,Y) = \frac{1}{N} \sum_{i=1,N} \left(\frac{X_i - X_{offset}}{\phi_X} \right) \left(\frac{Y_i - Y_{offset}}{\phi_Y} \right)$$

where

$$\phi_G = \sqrt{\sum_{i=1,N} \frac{(G_i - G_{offset})^2}{N}}$$

When G_{offset} is set to the mean of observations on G, then ϕ_G becomes the standard deviation of G, and S(X,Y) is exactly equal to the Pearson correlation coefficient of the observations of X and Y (Eisen *et al.*, 1998).

Graphical Representation. The hierarchical clustering algorithm used is based on the average-linkage method, which was developed for clustering correlation matrices such as those from microarray (Sneath and Sokal, 1973). The goal is to compute a dendogram that assembles all elements into a single tree. For any set of n genes, an upper-diagonal similarity matrix is computed by using the metric just described, which contains similarity scores for all pairs of genes. The matrix is scanned to identify the highest value (representing the most similar pair of genes.) A node is created joining these two genes, and a gene expression profile is computed for the node by averaging observation for the elements (missing values are omitted and the two joined elements are weighed by the number of genes they contain). The similarity matrix is updated with this new node replacing the two joined elements, and the process is repeated n-1 times until only a single element remains (Eisen *et al.*, 1998).

The clustered data are represented graphically by coloring each cell on the basis of the measured ratio to a common reference. Cells with log ratios of 0 are colored black, increasingly positive log ratios with reds of increasing intensities, and increasingly negative log ratios with greens of increasing intensity. The end product is a simple representation of genomic expression pattern (Eisen *et al.*, 1998).

Results

Eliminating Error

Normalization. Initial experiments showed that reproducibility is achievable only with sequences in the high signal intensity range (Figure 5-3). Furthermore, the specified control sequences on the arrays did not hybridize well with ^{33}P-labelled cDNA probes from LNCaP cells. The low level of hybridization for control sequences introduced variability such that ratios (control intensities/treated) calculated by the Research Genetics Pathways program had poor reproducibility.

For this reason, a new set of 30 control sequences in LNCaP and PC3 samples was identified (Table 5-1), which show stable expression across the dynamic range of signal intensities (Figure 5-4) for five hybridizations. Using a method similar to the algorithm used by Research Genetics, the intensities of the 30 sequences were summed (SI). An arbitrary high number was chosen (12×10^6) to obtain a factor of normalization (NF) for the remaining data points ($NF = 12 \times 10^6/SI$). SI and NF values were obtained for each hybridization experiment. To obtain normalized data for expression comparisons, raw intensity data points from each hybridization were multiplied by the corresponding factor NF to give the normalized signal (NS). Ratios of NS were used for comparison of expression.

Replicates. In LNCaP cells, the data were obtained from 3 replicate hybridizations with RNA from DMSO -treated cells, and 2 from genistein (37 μM)-treated cells, 2 from genistein (111 μM)-treated cells, 2 from biochanin (37 μM)-treated cells, and 2 from biochanin (111 μM)-treated cells. In PC-3 cells, the data were obtained from 3 replicate hybridizations with RNA from DMSO -treated cells, 2 from genistein (111 μM)-treated cells, and 2 from biochanin (111 μM)-treated cells. Ratios for control/treatment levels were then calculated from normalized signal intensities over the average of replicates.

Selection and narrowing the list of genes. Of 3500 distinct cDNA sequences represented on the microarray, only those with reproducible expression patterns were analyzed (approximately 900). A second level of selection occurs where only the genes regulated two-fold or more, after treatment with a concentration of 37 μM genistein for 48 hours, were considered. Finally, using the top-down approach, known genes that are well-documented and involved in many key cellular functions were further considered.

Identification of Genes with Altered Expresion level

The average normalized gross intensities of replicates of each analyzed sample were calculated. A scatter plot of log-transformed gross signal intensities was then used to measure the expression level of the previously selected genes (approximately 900) between untreated and isoflavone-treated cancer cells. In this graph, the log-transformed average of the normalized gross intensities of one sample was plotted to the average of another sample. The vast majority of the genes examined were comparably expressed between untreated and isoflavone-treated human prostate LNCaP (Figure 5-5) and PC-3 cancer cells (Figure 5-6).

In LNCaP cells, using a 2-fold difference relative to untreated cells as the cut-off point, genistein at 37 μM altered the expression of 26 genes, with 17 upregulated and 9 downregulated (Figure 5-5A; Figure 5-7A). Biochanin, at 37 μM, changed the expression level of 16 genes, with 5 being upregulated and 11 downregulated (Figure 5-5B; Figure 5-7A). Furthermore, while genistein and biochanin affected the expression of distinct genes, they regulated shared groups of genes in similar manner, both at 37 μM and at higher cytotoxic concentrations (111 μM) (Figure 5-7B-D). There seems to be a unique molecular fingerprint associated with the individual isoflavones at their less cytotoxic concentration, which is different to the one at the cytotoxic one, as the groups of genes regulated at the 2 concentrations were different.

In PC-3 cells, using the same 2-fold difference relative to untreated cells, genistein (111 μM) altered the expression of 43 genes, with 15 upregulated and 28 downregulated (Figure 5-6A; Figure 5-7E). Biochanin (111 μM) modified the expression of 40 genes, with 32 upregulated and 8 downregulated (Figure 5-6B; Figure 5-7E). The two isoflavones had effects on shared and distinct groups of genes at their lower cytotoxic concentrations (Figure 5-7E).

Cellular Functions Targeted by Genistein (37 μM) in LNCaP Cells

Genistein was found to alter 2-fold or more the expression of genes from a variety of cellular functions including signal transduction, transcription, and protein degradation (Table 5-2; Table 5-3).

Signal transduction. Two genes involved in signal transduction were detected only in treated cells. These genes were tyrosine kinase with EGF homology domains, and

interleukin-1 receptor associated protein. The expression of solute carrier 5 (inositol transporters) was not observed following genistein treatment.

Trancription. Four genes that participate in transcription were only detectable in genistein-treated cells. These genes were fibrillarin, amino-terminal enhancer of split, and TAR RNA-binding protein 2. Nuclear RNA-binding protein, another transcription gene was however downregulated 4.9-fold with genistein.

Protein degradation. One of the key enzymes of in the ubiquitin pathway (ubiquitin conjugating enzyme, UBC4) was downregulated 2.8-fold with genistein. This pathway is involved in the degradation of many short-lived cell cycle regulatory proteins, including p53 tumor suppressor protein.

Cellular Functions Targeted by Biochanin A (37 μM) in LNCaP Cells

Treatment of LNCaP cells with biochanin A for 48 hours modified 2-fold or more the expression of a number of genes involved in multiple cellular functions, including signal transduction, transcription, translation and cell adhesion (Table 5-4; Table 5-5).

Signal transduction. The expression of the gene for guanine nucleotide binding protein was 2-fold lower with biochanin A.

Transcription. This cellular process was affected with NFK-B being downregulated. NFK-B is known to regulate the expression of genes involved in cell proliferation and cell survival. Other genes, downregulated and involved in this cellular activity, include splicing factor and nuclear RNA-binding protein. Fibrillarin was upregulated.

Translation. Two genes that are involved in translation and protein processing were downregulated, namely prefoldin 5 and eukariotyc translation initiation factor 5.

Cell adhesion. The expression of cadherin 2 was no longer detectable in biochanin-treated cells, suggesting an effect on cell adhesion.

Cellular Functions Targeted by Genistein (111 μM) in PC3 Cells

In PC-3 cells, genistein modified 2-fold or more the expression level of a number of genes involved in distinct cellular activities, including signal transduction, cell survival, growth inhibition, transcription, DNA synthesis, translation, and protein degradation (Table 5-6; Table 5-7).

Signal transduction. Signal transduction in genistein-treated PC3 cells may be disrupted in that 7 genes (including epidermal growth factor receptor, mitogen-activated protein kinase-activated protein kinase 2) were downregulated by more than 3-fold. The other genes found to be altered were protein tyrosine phosphatase type IVA and type 2, protein kinase c-delta, phosphoinositide-3-kinase, and dual specificity phosphatase 11. One gene was, however, upregulated by genistein treatment, which is Ran GTPase activating protein 1.

Cell survival. While 2 genes involved in cell survival were upregulated, 6 genes (including prostate tumor overexpressed gene 1, delayed-early response gene 12, TGFB-1-induced antiapoptotic factor 1, granulin, skb1 homolog) that support cell survival were downregulated with genistein.

Growth inhibition. Furthermore, 3 genes whose expression promotes cellular growth inhibition (TGFβ, IGF binding protein 6, and cell division cycle 2-like 1) were upregulated.

Transcription. RNA processing was also affected as genes involved in this cellular function were both upregulated (3 genes), and downregulated (7 genes). The

transcription regulatory genes upregulated were fibrillarin, SNRP, and heterogenous nuclear ribonucleoprotei. Some of the transcription regulatory genes, downregulated by genistein, included RNA polymerase II, DR1-associated protein, general transcription factor IIIC, and adenosine deaminase.

DNA synthesis. It is not surprising that 2 genes that control DNA synthesis were downregulated since genistein completely blocks the incorporation of ^{3}H-thymidine in these cells at 111 μM, after 48 hours.

Translation. Three genes involved in translation were upregulated, which were CAAX box 1, ribosomal protein S29 and ribosomal protein L6. In addition, three other genes, that take part in translation, were downregulated, which were glutamyl-prolyl-tRNA synthetase, anchor attachment protein 1, and phenylalanine-tRNA synthetase-like.

Protein degradation. Genistein altered the expression of 6 genes involved in this cellular activity, with 3 being upregulated and 3 downregulated. The genes upregulated were ubiquitin conjugating enzyme E2D2, protease sorting nexin 1. The genes downregulated were proteosome subunit beta type 6, ubiquitin conjugating enzyme E2N, and F-box/W40 domain protein 1B.

Cellular Functions Targeted by Biochanin A (111 μM) in PC3 Cells

Biochanin changed 2-fold or more the expression level of a group of genes involved in multiple cellular activities, including signal transduction, cell survival, growth inhibition, transcription, DNA synthesis, translation, protein degradation, and cell adhesion (Table 5-8; Table 5-9).

Signal transduction. Contrary to genistein, the effects of biochanin A (111 μM) on signal transduction were for the most part upregulation (11 upregulated, and 2

downregulated). Genes that were upregulated include dual specificity phosphotase 5, protein phosphatase 4, MKP-1 like protein tyrosine phosphatase and MEK partner 1. The genes that were downregulated were phosphoserine phosphatase-like, and dual specificity phosphatase 11.

Cell survival. Genes that contribute to cell survival were both upregulated (2 genes) and downregulated (1 gene). Nucleophosmin and defender against cell death 1 were upregulated. However, prostate tumor overexpressed gene 1 was downregulated.

Growth inhibition. Three genes involved in growth inhibition were only upregulated. These genes were IGFBP6, TGFβ, and cell division cycle 2 like 1.

Transcription. Biochanin upregulated 8 genes that control RNA processing, with 7 being upregulated. These upregulated genes included transcription factor 2 CP2, fibrillarin, and AE-binding protein 1. RNA polymerase II was, however, downregulated.

DNA synthesis. Two genes involved in DNA synthesis were downregulated by biochanin treatment. These included primase and APEX nuclease (multifunction DNA repair enzyme).

Translation. Synthesis of new proteins was also affected in that 3 genes involved in protein processing were upregulated (including ribosomal protein L6, and CAAX box 1). Furthermore, phenylalanine-tRNA synthetase-like was downregulated.

Protein degradation. The half-life of many cellular proteins may be modified as genes that control protein degradation had their expression levels altered by biochanin A. These effects were the upregulation of 5 genes involved in this process (including ubiquitin conjugating enzyme E2D2, ubiquitin thiolesterase, and cysteine protease). In addition, the gene coding for the proteosome subunit beta type 6 was downregulated.

Cell adhesion. Biochanin treatment upregulated the expression of integrin alpha V, which is a key protein involved cell adhesion.

Clustering of Genes and Hybridizations

The samples analyzed were RNA from normal human prostate tissue (2), DMSO-treated LNCaP (3), genistein (37 μM)-treated LNCaP (2), genistein (111 μM)-treated LNCaP (2), biochanin (37 μM)-treated LNCaP (2), biochanin (111 μM)-treated LNCaP (2), DMSO-treated PC-3 (3), genistein (111 μM)-treated PC-3 (2), biochanin (111 μM)-treated PC-3 (2). Average hybridizations of each treatment condition were clustered. Individual experiments were clustered according to the cell line (Figure 5-8). This is not surprising since the fraction of genes regulated by the isoflavones is small enough not to alter the molecular profile of the cell line. Furthermore, graphical representation of clustered genes (Figure 5-9) reveals 24 main clusters with correlation coefficients between 0.85-0.96 with varying number of genes. Some of the clusters with the highest correlation coefficents (cc) include cluster O (6 genes) with cc = 0.93, which contains the gene for TGFβ (Figure 5-10A), cluster P (10 genes) with cc = 0.95, which contains the genes for lamin and keratin (Figure 5-10B), cluster S (17 genes) with cc = 0.96, which contains the gene for laminin receptor (Figure 5-10C).

Discussion

The isoflavonoids genistein and biochanin A have been shown to exhibit antiproliferative effects on the human prostate cancer cell lines LNCaP and PC3. A number of key cellular targets have been identified including the cell cycle regulatory proteins cyclin B and $p21^{Cip1}$. The effects of these phytochemicals, however, probably

involve many genes and signal pathways controlling cell proliferation, DNA synthesis, progression through the cell cycle and other cellular activities. The advent of microarray technology permits us to obtain the patterns of gene expression (on a more global scale) (Brown and Botstein, 1999) that arise in the cells following treatment with these isoflavonoids. Microarray technology has already been used to assess gene expression profiles of number of human cancer cell lines including prostate cancer (Bubendorf *et al.*, 1999; Scherf *et al.*, 2000), and to obtain molecular portraits of tumors from patients with breast cancer (Perou *et al.*, 1999, 2000). Furthermore, the application of DNA array technology was recently used to screen the effects of thalidomide in LNCaP cells (Dixon *et al.*, 1999). Although microarray hybridizations may be perceived as a simple scaling up (in number of genes), miniaturization (in array platform size) and automation of hybridization measurements that have been standard for many years, the sheer volume of the data from microarray analysis yields much more than just more data, but a global view of how genes from multiple cellular pathways are connected. The body of expression data obtained here shows how the individual isoflavone affects distinct groups of genes from multiple cellular functions. Thus, the integrated effects on these various cellular activities may partly explain the net biological effects of these compounds in the prostate cancer cells. At their lower cytotoxic concentration, each isoflavone exhibits a unique molecular fingerprint as each regulated distinct groups of genes in either cell lines. Furthermore, in LNCaP cells, there seems to be concentration-specific molecular fingerprints, as genistein and biochanin did not regulate the same sets of genes at 37 μM compared with 111 μM.

The information obtained here from the microarray analysis supports previous data in that the isoflavonoids do affect the cell proliferation of prostate cancer cells in a well-defined way, yet through a variety of mechanisms (Adlercreutz *et al.*, 1992). In LNCaP cells, genistein and biochanin A upregulate and downregulate various components of cellular signal transduction which may suggest some inhibiting pathways may be activated, while other signals for growth may be suppressed. In many signal transduction pathways, the net result is an effect on transcription. As expected, many transcription regulatory genes were either turned on or off. The next step after transcription is translation, and biochanin A does seem to downregulate genes that participate in protein processing. In PC3 cells, similar observations are reported where genistein and biochanin A alter the expression of genes involved in signal transduction, cell survival, transcription and translation. Furthermore, both chemicals upregulate genes that promote growth inhibition. These results support the idea that the antiproliferative effects of these isoflavonoids are the products of changes in gene expression from a variety of cellular activities.

While DNA microarray technology offers great promise for discovering cellular targets of a given drug, it is still at its infancy and has certain limitations. Microarray output is subject to recognized variability (Duggan *et al.*, 1999; Lee *et al.*, 2000). When using radiolabeled probes with membrane-based array, serial or parallel hybridization is required, which can introduce the possibility of higher variability in comparison of expression levels. Furthermore, stripping of membranes for multiple uses may decrease the efficiency of subsequent hybridizations. When extracting data from phosphor-image representations of radioactive hybridizations, there can be difficulties with analysis.

There can be non-linear warping of the membrane, which means that the observed array may not have the strict geometric regularity. This introduces difficulty in obtaining accurate grids to specify target locations.

Variations may occur from the samples themselves. For the most part, experimental procedures with cells in culture can be very reproducible; however, little is known about the global expression of genes of a cell line from different passages. A number of spots on the membrane will yield strong signals following hybridizations with radiolabeled probes. The spots that have weak signals, however, may be so because of reasons other than their actual expression. For example, low abundance mRNA species (approximately 1 transcript per 100,000) may not sufficiently interact with the target sequence on the membrane in order to hybridize (Southern *et al.*, 1999). If there is hybridization, the signal can be so weak that it is rendered undetectable above assay noise. Sequences hybridized at the low signal intensities tend to have poor reproducibility.

The approach described in this chapter to discover gene targets by the isoflavonoids serves a number of functions. First, it does support previous data in terms of how these phytochemicals affect multiple cellular activities, in order to elicit growth arrest in the prostate cancer cells. Second, it suggests possible novel gene targets, thus shedding light on alternative modes of action, and on how the complex cellular and biochemical pathways are interrelated. In LNCaP cells, genistein downregulated nuclear receptor subfamily 2. This gene codes for a steroid hormone receptor, and its downregulation may have significance for a hormone-sensitive prostate cancer cells. Biochanin, in LNCaP cells, downregulated the gene for EGF-R pathway substrate 15,

which may indicate an effect of this isoflavone on an element of downstream signal transduction for this receptor. In PC-3 cells, genistein downregulated the genes for EGF-R and MAPKK. This effect suggests that genistein can alter signal transduction by directly downregulating the expression of the involved genes. Biochanin, in PC-3 cells, upregulated a negative regulator of cell proliferation, TGFβ. These genes represent possible new targets of the isoflavones.

The two prostate cancer cell lines have distinct gene expression profiles. This difference in gene expression pattern may explain the differential sensitivity of these cell lines to treatment with the isoflavonoids. In a screening of potential anticancer drugs by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI), it was shown that cancer cell lines can to be clustered by their sensitivity to various drugs (Weinstein *et al.*, 1997). Furthermore, a particular group of drugs tends to affect cancer cells with similar expression patterns, as seen by cDNA microarray analysis (Scherf *et al.*, 2000). Cancer cells with similar p53 status had similar sensitivity to anticancer drugs (Scherf *et al.*, 2000). The p53 status between LNCaP and PC-3 cells may explain the differential effects of the isoflavonoids, including the resulting gene exprssion profile as p53 regulates a number of genes.

While interesting data are emerging, there are still lingering questions: what is the validity and quantitative accuracy of the observed genes? Which genes should be queried further? What gene changes reflect the mechanisms of actions and which are just consequences?

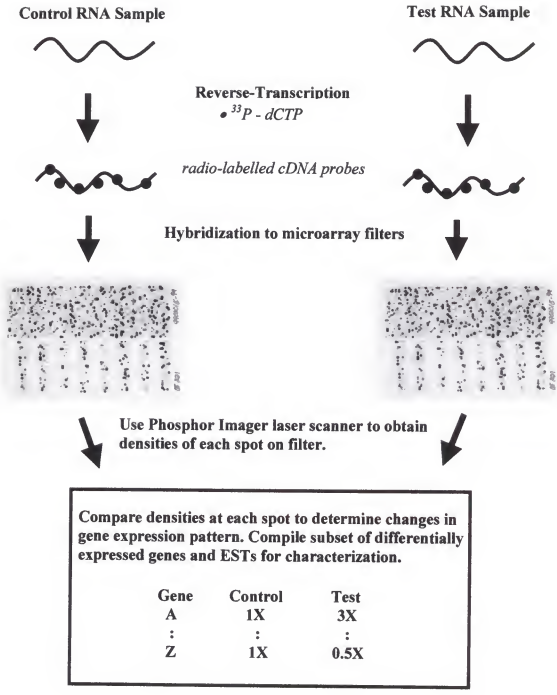


FIGURE 5-1. Principle of DNA microarray technology.

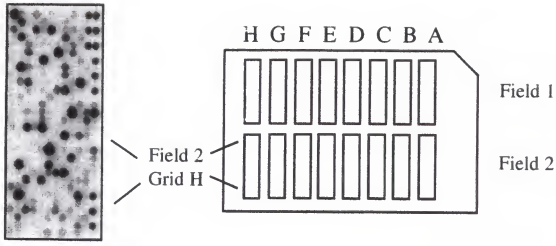


FIGURE 5-2. Organization of imprinted sequences on the microarray membranes

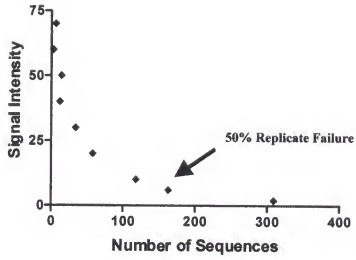


FIGURE 5-3. Correlation between signal intensity and number of sequences. Data from sequences hybridized in the cDNA microarrays at low intensities become less reproducible.

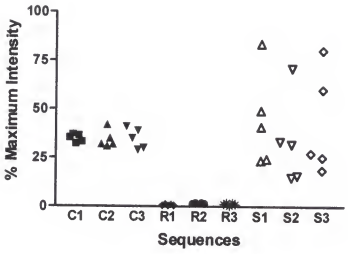


Figure 5-4. Control sequences. A subset of sequences was identified which had stable expression across hybridizations. C1-C3 are representratives of the 30 sequences selected for normalization. R1-R3 are control sequences from the manufacturer. S1-S3 are representatives of other sequences on the membrane.

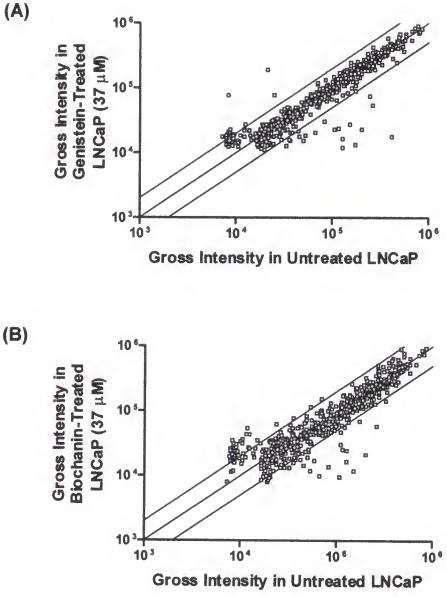


FIGURE 5-5. Scatter plot of log-transformed gross intensity for 870 genes expressed in isoflavone-treated LNCaP cells. The central diagonal line represents the line of identity (untreated/treated = 1). The two outer parallel lines represent two-fold difference in mRNA levels (A) between untreated and genistein-treated, (B) between untreated and biochanin-treated LNCaP prostate cancer cells.

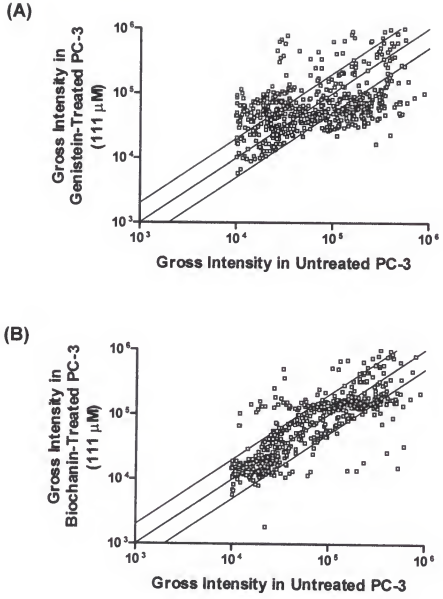


FIGURE 5-6. Scatter plot of log-transformed gross intensity for 870 genes expressed in isoflavone-treated PC-3 cells. The central diagonal line represents the line of identity (untreated/treated = 1). The two outer parallel lines represent two-fold difference in mRNA levels (A) between untreated and genistein-treated, (B) between untreated and biochanin-treated PC-3 prostate cancer cells.

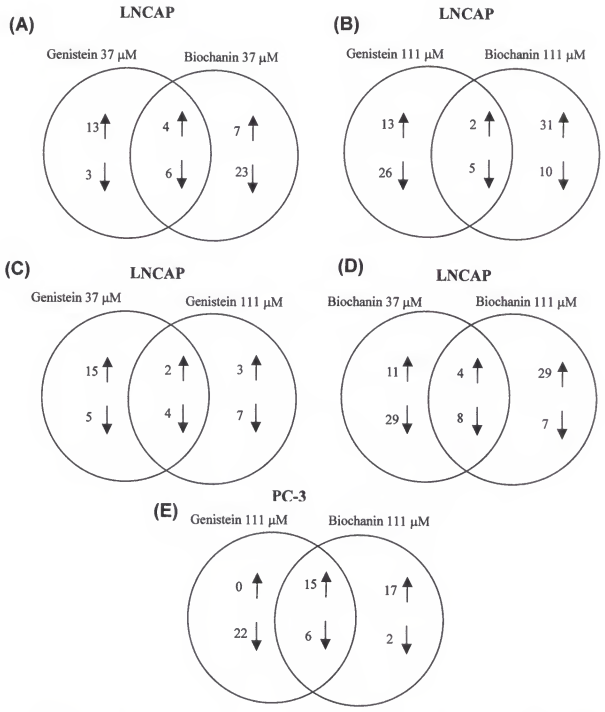


FIGURE 5-7. Differential effects on gene expression pattern by the isoflavonoids. Genistein and biochanin affect similar and different groups of genes at their cytostatic doses (A) and at their cytotoxic doses (B) in LNCaP cells. The isoflavonoids genistein (C) and biochanin (D) alter similar and different groups of genes between their cytostatic and cytotoxic doses. In PC-3 cells (E), genistein and biochanin change the expression pattern of similar and different groups of genes.

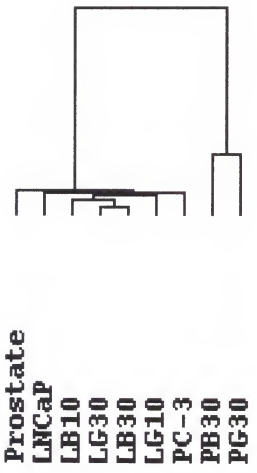


FIGURE 5-8. Clustering of average microarray hybridizations for each experiment. LB10 = biochanin-treated LNCaP (37 μM). LB30 = biochanin-treated LNCaP (111 μM). LG10 = genistein-treated LNCaP (37 μM). LG30 = genistein-treated LNCaP (111 μM). PB30 = biochanin-treated PC-3 (111 μM). PG30 = genistein-treated PC-3 (111 μM).

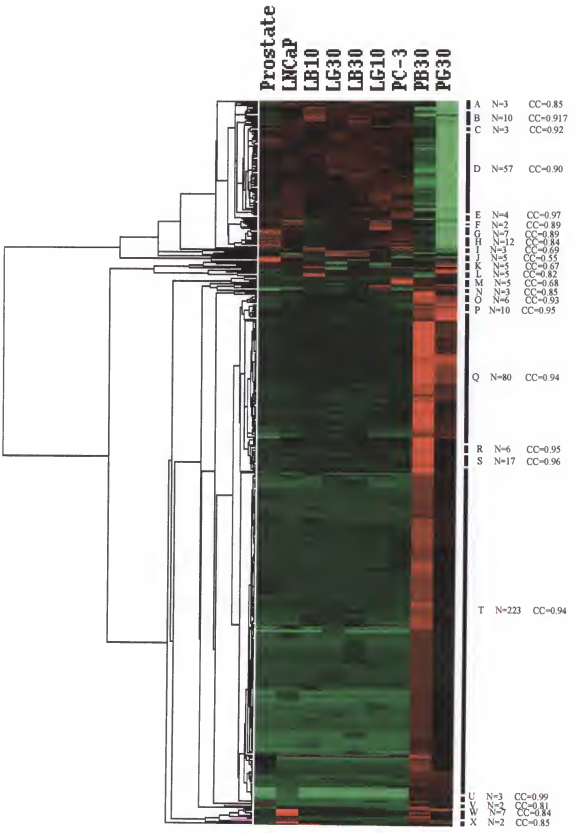
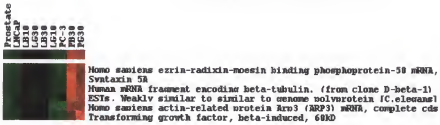


FIGURE 5-9. Clustering of genes from all hybridizations. Clusters (A-X) are shown on the right, where N is the number of genes, and CC is the correlation coefficient of the cluster. ■ Positive ■ Negative ■ Zero ■ Missing

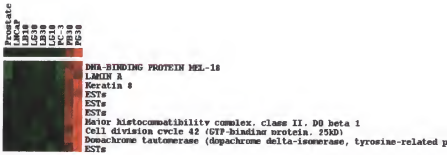
A)

Cluster O
Correlation Coefficient = 0.93



B)

Cluster P
Correlation Coefficient = 0.95



C)

Cluster S
Correlation Coefficient = 0.96

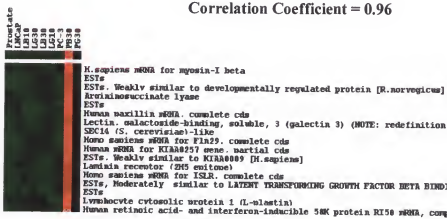


FIGURE 5-10. Selected clusters. Correlation of coefficient A) 0.94 B) 0.95 C) 0.96 clustered with ubiquitin conjugating enzyme.

TABLE 5-1. List of control genes.

GENE NAME*	ACCESSION #
Myosin Light Chain Alkali, Smooth Muscle Isoform	AA88346
Thymosin Beta-10	AA486085
Villin 2(ezrin)	AA411440
GCN5-like 1	H94857
Dopachrome Tautomerase	AA397824
ATP Synthase Lipid-Binding Protein P1 Precursor	AA046701
MHC Class I Protein HLA-A (HLA-A28, -B40, -Cw3)	AA644657
Proteasome (prosome, macropain) subunit, beta type, 6	AA070997
Human Glutathione-S-Transferase Homolog mRNA cds	AA441895
Transforming Growth Factor, beta-induced	AA633901
H. Sapiens mRNA for ubiquinol-cytochrome c reductase	AA629862
H. Sapiens mRNA for L-3-phosphoserine-phosphatase	N75028
H. Sapiens mRNA for procesing a-glucosidase I	AA291490
H. Sapiens mRNA for ITBA1 protein	AA455272
H. sapiens (clone mf.18) RNA polymerase II mRNA	AA460830
Human mRNA for KIAA0228 gene	AA431206
Human mRNA for pM5 protein	AA629923
ESTs, Highly Similar to Integral Membrane Protein E16	AA419177
ESTs, Moderately Similar to Gamma-Adaptin	N78621
ESTs, Weakly Similar to H. Sapiens KIAA0108	AA600214
ESTs, Weakly Similar to Hypothetical Protein Secb-Tdh	R94693
ESTs	AA056484
ESTs	AA485877
ESTs	N2598
ESTs	AA150979
ESTs	AA025746
ESTs	W63789
ESTs	R68464
ESTs	AA015607
ESTs	R69566

* Gene name determined from Unigene Cluster I.D.

TABLE 5-2. Genestein (37 μM)-upregulated genes in LNCaP cells.

GENE NAME*	ACC #	DMSO	Genistein (fold change)	Cellular Function
Tyrosine kinase with EGF homology domains	AA432062	-	+	Signal Transduction
Interleukin-1 receptor associated protein	AA121428	-	+	
Protein phosphatase 3	AA457092	-	+	
Amino-terminal enhancer of split	AA485742	-	+	Transcription RNA Processing
TAR RNA-binding protein 2	AA436409	-	+	
Fibrillarin	*AA663986*	-	+	
Emopamil-binding protein	N67038	-	+	Other
Ral binding protein 1	AA085619	-	+	
Homo sapiens mRNA	*AA173611*	-	+	
Mannosidase	AA427691	-	+	
t-complex 1	R46821	-	+	
mRNA sequence/transcript KIAA0493	R61518	-	+	
Crystallin	AA504891	-	10	

* Gene name determined from Unigene Unigene Cluster I.D.
Italic means it is also regulated by biochanin in the same cell line.

TABLE 5-3. Genistein (37 μM)-downregulated genes in LNCaP cells.

GENE NAME*	ACC #	DMSO	Genistein (fold change)	Cellular Function
Solute carrier 5 (inositol transporters)	AA490044	+	-	Signal Transduction
Nuclear receptor subfamily 2	AA666180	+	-	
Solute carrier 25	AA486200	+	-5.0	
non-Pou domain-containing binding protein	*AA056465*	+	-	Transcription
Nuclear RNA-binding protein	*AA056465*	+	*-4.9*	RNA Processing
Ubiquitin-conjugating enzyme E2D2	*AA431869*	+	*-2.8*	Protein Degradation
Glutamate dehydrogenase	R54424	+	-	Other
NADH dehydrogenase (ubiquinone)	AA608515	+	-	

* Gene name determined from Unigene Cluster I.D.
Italic means it is also regulated by biochanin in the same cell line.

TABLE 5-4. Biochanin (37 μM)-upregulated genes in LNCaP cells.

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
SH3-domain GRB2-like 1	AA398366	-	+	Signal Transduction
Fibrillarin	*AA663986*	-	+	Transcription RNA Processing
mRNA sequences/ transcript KIAA0493	*R61518*	-	+	Other
KIAA0376 protein	R43372	-	+	
Homo sapiens mRNA; cDNA DKFZp586N012	*AA173611*	-	+	
KIAA0247 gene product	AA451632	-	+	
Capping protein	AA430524	-	+	
Homo sapiens clone 24703 beta-tubulin mRNA	AA427899	+	2.0	
TNFRSF1A modulator	AA479741	+	2.0	

* Gene name determined from Unigene Cluster I.D.
Italic means it is also regulated by genistein in the same cell line.

TABLE 5-5. Biochanin (37 μM)-downregulated genes in LNCaP cells.

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
EGF-R pathway substrate 15	AA490223	+	-	Signal Transduction
Guanine nucleotide binding protein	AA490256	+	-2	
Splicing factor	T65786	+	-2.6	Transcription RNA Processing
Nuclear factor Kappa-B, subunit 3	AA443547	+	-2.2	
Nuclear RNA-binding protein	*AA056465*	+	*-4.6*	
Prefoldin 5	AA446453	+	-5.5	Translation Protein Processing
Eukaryotic translation initiation factor 5	*AA669443*	+	-2.3	
Ubiquitin-conjugating enzyme E2D2	*AA431869*	+	*-6.1*	Protein Degradation
Cadherin 2	W49619	+	-	Cell Adhesion
NADH dehydrogenase	*AA608515*	+	-	Other
Glutathione peroxidase 1	AA485362	+	-	
Folate receptor	*R24530*	+	-23.5	
GM2 ganglioside activator protein	*AA4553471*	+	-11.2	
Homo sapiens mRNA for leptin receptor	H51066	+	-2.6	
Antiquitin 1	AA102646	+	-2.2	
Homo sapiens agrin precursor mRNA	*AA458878*	+	-3.7	
Chromosome 18 open reading frame 1	AA489633	+	-2	

TABLE 5-5. Continued

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
Solute carrier 25	*AA486200*	+	4.6	Other

* Gene name determined from Unigene Cluster I.D.
Italic means it is also regulated by genistein in the same cell line.

TABLE 5-6. Genistein (111 μM)-upregulated genes in PC3 cells.

GENE NAME*	ACC #	DMSO	Genistein (fold change)	Cellular Function
Ran GTPase activating protein 1	*AA485734*	+	*2.7*	Signal Transduction
Nucleophosmin	*AA669758*	+	*3.0*	Cell Survival
Defender against cell death 1	*AA455281*	+	*4.9*	
Insulin-like growth factor binding protein 6	*AA478724*	+	*5.7*	Growth Inhibition
Transforming growth factor beta	*AA633901*	+	*2.5*	
Cell division cycle 2-like 1	*AA443050*	+	*3.3*	
Fibrillarin	*AA663986*	-	+	Transcription RNA Processing
Small nuclear ribonucleoprotein polypeptide A'	*AA122272*	+	*2.6*	
Heterogenous nuclear ribonucleoprotein U	*AA496741*	+	*4.8*	
CAAX box 1	*W72596*	+	*3.1*	Translation Protein Processing
Ribosomal protein S29	*AA411343*	+	*2.2*	
Ribosomal protein L6	*AA629808*	+	*3.3*	
Protease, cysteine	*AA425938*	+	*2.0*	Protein Degradation
Ubiquitin conjugating enzyme E2D2	*AA431869*	-	+	
Sorting nexin 1	*AA449430*	+	*3.5*	

* Gene name determine from Unigene Cluster I.D.
Italic means it is also regulated by biochanin in the same cell line.

TABLE 5-7. Genistein (111 μM)-downregulated genes in PC3 cells.

GENE NAME*	ACC #	DMSO	Genistein (fold change)	Cellular Function
Protein tyrosine phosphatase, receptor type 2	AA464542	+	-3.3	Signal Transduction
Epidermal growth factor receptor	W48713	+	-3.4	
Protein tyrosine phosphatase type IVA	AA039851	+	-3.4	
Protein kinase c-delta, ankyrin repeat domain 3	W72972	+	-3.8	
Mitogen-activated protein kinase-activated protein kinase 2	AA455056	+	-5.5	
Phosphoinositide-3-kinase, polypeptide 1	AA463460	+	-5.9	
Dual specificity phosphatase 11 (RNA/RNP complex)	*AA463480*	+	*-9.9*	
Delayed-early response gene 12	AA402891	+	-3.7	Cell Survival
TGFB-1-induced antiapoptotic factor 1	AA446223	+	-2.6	
Granulin	AA431832	+	-2.6	
Skb1 homolog, shk1 kinase-binding protein 1	AA496357	+	-3.9	
Transmembrane 4 superfamily member 6	H87106	+	-5.3	
Prostate tumor overexpressed gene 1	*AA488053*	+	*-6.8*	
TBP-associated factor 1C, RNA polymerase I	AA454218	+	-2.8	Transcription RNA Processing
DR1-associated protein, DRAP1	AA406285	+	-3.4	
General transcription factor IIIC	R43008	+	-3.5	
Adenosine deaminase, RNA-specific	AA600189	+	-3.9	
Zing finger protein 42	AA120779	+	-4.7	
RNA polymerase II (DNA directed), polypeptide A	AA479052	+	-5.0	
RNA polymerase II (DNA directed) poylpeptide	*AA027042*	+	*-4.0*	
Primase, polypeptide 2A	*AA434404*	+	*-3.3*	DNA Synthesis
APEX nuclease (multifunction DNA repair enzyme)	*AA478273*	+	*-6.6*	
Glutamyl-prolyl-tRNA synthetase	AA599158	+	-3.0	Translation Protein Processing
Anchor attachment protein 1	AA455301	+	-4.7	
Phenylalanine-tRNA synthetase-like	*W96450*	+	*-2.5*	

TABLE 5-7. Continued

GENE NAME*	ACC #	DMSO	Genistein (fold change)	Cellular Function
Ubiquitin conjugating enzyme E2N	AA490124	+	-3.1	Protein Degradation
F-box and WD-40 domain protein 1B	H97827	+	-5.0	
Proteosome subunit, beta type 6	*AA070997*	+	*-5.3*	

* Gene name determine from Unigene Cluster I.D.
Italic means it is also regulated by biochanin in the same cell line.

TABLE 5-8. Biochanin (111 μM)-upregulated genes in PC3 cells.

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
Ephrn-B2	AA461424	+	5.8	Signal Transduction
Dual specificity phosphatase 5	W65461	+	5.4	
Tensin	H10988	+	5.4	
MKP-1 like protein tyrosine phosphatase	AA129677	+	5.1	
Ribosomal protein S6 kinase	AA452574	+	3.9	
Protein phosphatase 4	AA405562	+	3.7	
TRAF family-associated NFKB activator	AA134814	+	3.3	
Phosphotase and tensin homolog	W37864	+	3.1	
MEK partner 1	W19601	+	2.4	
Epidermal growth factor receptor pathway substrate 15	AA490223	+	2.3	
Ran GTPase activating protein 1	*AA485734*	+	*3.6*	
Nucleophosmin	*AA669758*	+	*4.8*	Cell Survival
Defender against cell death 1	*AA455281*	+	*5.4*	
Insulin-like growth factor binding protein 6	*AA478724*	+	*6.3*	Growth Inhibition
Transforming growth factor beta	*AA633901*	+	*2.1*	
Cell division cycle 2-like 1	*AA443050*	+	*6.5*	
Fibrillarin	*AA663986*	-	+	Transcription RNA Processing
Small nuclear ribonucleoprotein polypeptide A'	*AA122272*	+	*3.8*	
Heterogenous nuclear ribonucleoprotein U	*AA496741*	+	*4.9*	
Transcription factor 2 CP2	AA488618	+	5.6	
AE-binding protein 1	AA490462	+	4.4	
Heterogenous nuclear ribonucleoprotein H1	W96058	+	3.0	
non-POU-domain-containing, octamer binding	AA056465	+	2.8	

TABLE 5-8. Continued.

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
CAAX box 1	*W72596*	+	*6.8*	Translation Protein Processing
Ribosomal protein S29	*AA411343*	+	*4.2*	
Ribosomal protein L6	*AA629808*	+	*10.2*	
Ubiquitin thiolesterase	AA670438	+	4.5	Protein Degradation
Cathepsin D, lysomal proteinase	AA485373	+	3.8	
Protease, cysteine	*AA425938*	+	*4.2*	
Ubiquitin conjugating enzyme E2D2	*AA431869*	-	+	
Sorting nexin 1	*AA449430*	+	*7.8*	
Intergrin alpha V	AA029934	+	3.7	Cell Adhesion

* Gene name determined from Unigene Cluster I.D.
Italic means it is also regulated by genistein in the same cell line.

TABLE 5-9. Biochanin (111 μM)-downregulated genes in PC3 cells.

GENE NAME*	ACC #	DMSO	Biochanin (fold change)	Cellular Function
Phosphoserine phosphotase-like	N75028	+	-2.6	Signal Transduction
Dual specificity phosphatase 11 (RNA/RNP complex)	*AA463480*	+	*-2.8*	
Prostate tumor overexpressed gene 1	*AA488053*	+	*-3.4*	Cell Survival
				Growth Inhibition
RNA polymerase II (DNA directed) poylpeptide E	*AA027042*	+	*-2.0*	Transcription RNA Processing
Primase, polypeptide 2A	*AA434404*	+	*-3.1*	DNA Synthesis
APEX nuclease (multifunction DNA repair enzyme)	*AA478273*	+	*-2.7*	
Phenylalanine-tRNA synthetase-like	*W96450*	+	*-2.2*	Translation Protein Processing
Proteosome subunit, beta type 6	*AA070997*	+	*-2.2*	Protein Degradation
Intergrin alpha V	AA029934	+	3.7	Cell Adhesion

* Gene name determined from Unigene Cluster I.D.
Italic means it is also regulated by genistein in the same cell line.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

A chronic degenerative disease like prostate cancer has multifactorial origin. An intricate network connects this disease with multiple risk factors and also with multiple protective factors. From the point of view of preventive medicine, this implies that removal of a single risk factor will have beneficial impact on the epidemiology of prostate cancer. Similarly, a single protective factor can decrease the risk of developing this clinical condition. Because of its extended preclinical phase or latency, prostate cancer offers a wide window of opportunity for a protective agent to inhibit its clinical progression by interfering negatively with the carcinogenesis process (Figure 6-1). Epidemiology studies of prostate cancer have identified one such potential family of protective agents, namely the isoflavonoids. Of course, a rational use of chemopreventive agents is based not only on the assessment of their efficacy and safety, but also on understanding of their mechanisms of action.

This study investigated the antiproliferative effects and the mechanisms of action of two potent isoflavonoids, genistein and biochanin in human prostate cancer cells. Both compounds decreased, in a dose-dependent fashion, cell viability, and DNA synthesis in LNCaP (androgen-sensitive, early clinical stage) and in PC-3 (androgen-independent, late clinical stage) prostate cancer cells. These effects have been reported in the literature, in other human cancer cell lines, including in prostate cancer cells. However, only the androgen-sensitive cells underwent apoptosis in a dose-dependent

manner with both genistein and biochanin. PC-3 cells were resistant to DNA fragmentation indicative of apoptosis. These cells may undergo different forms of cell death, including autophagy where the degradation of cellular organelles occurs, with conserved cytoskeleton structure and intact genomic DNA. Future experiments should examine possible synergestic or additive effects of genistein and biochanin A where inhibition might occur at lower doses of each individual compound. In addition, future studies should focus on identifying the mode of cell death that occurs in PC-3 cells with treatment of the isoflavonoids.

The phytochemicals interfered with cell cycle progression of the cancer cells. Genistein elicited a G2/M phase arrest in both LNCaP and PC-3 cells. Biochanin, however, induced a G2/M arrest only in PC-3 cells, which was similar to genistein. The number of cells increased in the G1 phase with treatment of biochanin in LNCaP cells. The effects on the cell cycle progression were probably the results of alteration in the protein expression level of key cell cycle regulatory proteins by the isoflavonoids. Cyclin A was lowered by both genistein and biochanin in LNCaP, and by biochanin in PC-3. Cyclin B protein was barely detectable with both chemicals in LNCaP cells, whereas in PC3 cells the decrease was observed, but less pronounced. Genistein elevated the $p21^{Cip1}$ protein level 2-fold in both cell lines, whereas biochanin lowered its level by 50% in both cell lines. The expression of cell cycle regulators p53 and p27 proteins was unchanged by both chemicals in LNCaP cells. Cyclin D levels were only only slightly lowered by genistein and biochanin in LNCaP cells. These effects on the cell cycle have been described elsewhere in other cell lines. Future experiments should investigate whether isoflavones alter the level of mRNAs of these cell cycle regulators. In addition, since the

effects on the cell cycle resemble those of known topoisomerase inhibitors (Del Bino *et al.*, 1991; Deptala *et al.*, 1999), and that the concentration range of genistein used in this study parallels the one at which it inhibits topoisomerase activity (Constantinou *et al.*, 1990; Markovits *et al.*, 1989), future experiments should investigate the effects on this enzyme.

The multiple effects of genistein and biochanin are due not only to their unique diphenolic structure, but also due to the functional groups attached to their carbon structure. Among the isoflavonoids screened, genistein and biochanin are the ones that have been consistently the most potent in their antiproliferative activities. They both share strong structural similarity with the only difference being a functional group on carbon 4' at which genistein has an hydroxy (-OH) group, and a methoxy ($-OCH_3$) is found in biochanin A (Figure 3-18; Figure 1-4). Daidzein also has a similar structure, but it lacks the hydroxy group on carbon 5, which is present in both genistein and biochanin (Figure 3-18; Figure 1-4). Thus, daidzein is missing the functional group that both genistein and biochanin have on this carbon. Coumestrol is not an isoflavone, but a coumestan, which belongs to the isoflavonoid family. Coumestrol, therefore, has the most dissimilar structure from the other isoflavonoids, and like daidzein, it is less effective than genistein and biochanin A (Figure 1-4). It appears, therefore, that the hydroxy group on carbon 5 of the isoflavone structure is necessary, and so are the functional groups (hydroxy or methoxy) attached to carbon 4', in order for these compounds to have the most potent anticancer properties. The diphenolic structure of the isoflavonoids shares similarity to that of estradiol, so future experiments on the structural

significance of these phytochemicals should include studying the effects of natural estrogen on the same endpoints investigated here.

Genistein has been used and described as a specific tyrosine kinase inhibitor, but the effects assessed in the prostate cancer cells can not be due solely to this activity. From a pharmacological perspective, the concentration of genistein used in this study is much lower than the dose at which its tyrosine kinase inhibition activity has been reported in other cells (Akiyama *et al.*, 1987). In LNCaP cells, inhibition of EGF-R tyrosine kinase was not observed with genistein and biochanin at concentrations that were inhibiting proliferation (Peterson and Barnes, 1993). Furthermore, another specific tyrosine kinase inhibitor, tyrphostin 25, was not able to duplicate at 250 μM the effects of either genistein or biochanin on cell viability, cell cycle phase distribution, and expression of cell cycle regulators in the prostate cancer cells. Genistein does not inhibit all tyrosine kinases, such as the insulin and the platelet derived growth factor receptors (Abler *et al.*, 1992; Davidai *et al.*, 1992). The receptor tyrosine kinase most inhibited by genistein is the epidermal growth factor receptor. This inhibition was not observed in whole cell, but it was reported with purified receptors (Akiyama *et al.*, 1987). Future experiments should include a determination of level of tyrosine phosphorylation in the prostate cancer cells at the concentration of genistein and biochanin used in this study, so as to completely rule out this mode of action in the effects reported here.

The isoflavonoids exhibit antitumorigenic effects. When athymic nude mice implanted with LNCaP tumors received biochanin for 10 days, their mean tumor volume was smaller compared to controls at 3 and 6 weeks. Tumor incidence was reduced with biochanin treatment at 3 weeks. When mice, with established tumors received genistein

or biochanin, a slower rate of growth was observed compared to controls. The dose used in the present animal study is a non-toxic dose that was reported for nude mice, based on body weight and general health (Yanagihara *et al.*, 1993). Therefore, future experiments should include the determination of a dose-response curve on the inhibitory effects of these chemicals in the nude mice. This would allow the identification of the maximum tolerated dose (MTD) and the minimum effective dose (MED). It would especially be advantageous to know the MED for each chemical, so that their synergistic or additive effects could be evaluated by administering both compounds to the mice at their lowest effective concentration. Furthermore, in order to gain insight in the mechanisms of action in vivo, tumors should be collected on the last day of treatment, and immunostained for cell cycle regulatory proteins, including cyclin B and $p21^{Cip1}$. The time of tumor collection is very crucial since at 3 weeks, in the present study, the cells in the tumor may be rebounding. Thus, the differences (if any) in proliferation or angiogenic markers may not be apparent between cells of tumors of treated and control mice at 3 weeks.

Future experiments should include immunohistochemical analysis of tumors at earlier time points, such as at 2, 4, 6, 8 and 10 days from the first day of treatment. The effects on proliferation and angiogensis markers may only be observed at these earlier points. The in vitro data showed LNCaP and PC-3 cells respond differentially to the isoflavonoids. A good model for the future is to implant PC-3 cells in the nude mice. These two cell lines have different status of p53, where this tumor suppressor is functional in LNCaP and non-functional in PC-3 cells. If PC-3 cells turn out to behave differently in the tumors of mice in response to isoflavone treatment, the animals should

be further implanted with p53-transfected PC-3 cells to test the role of this tumor suppressor protein in the antitumorigenic mechanisms of the phytochemicals. However, the status of p53 in prostate cancer patients is not a good marker for the progression of this disease (Downing *et al.*, 2001; Quinn *et al.*, 2000).

The cDNA microarray technology provided a genomic profile of the effects of genistein and biochanin in the prostate cancer cells. In LNCaP and PC-3 cells, genistein and biochanin altered expression of similar and unique genes involved in multiple cellular functions including DNA synthesis, transcription, translation, protein degradation, signal transduction, cell proliferation, and cell adhesion. The obvious future plan is to validate the changes of the individual genes identified as having altered expression before making any claims on possible mechanisms. However, certain possible novel target genes have been identified from the gene expression profile of cells treated with genistein and biochanin. These compounds affect shared, but also unique sets of genes in multiple cellular pathways at the gene expression level. Concentration-specific molecular fingerprints were identified for the individual isoflavones. The identification and the eventual validation, by Northern bloting or RT-PCR, of the targeted genes may provide clues to the cellular targets in cancer cells that are more vulnerable with chemopreventive agents, and possible lead to a better understanding of cancer cells and prevention.

Prostate cancer may be prevented not only by avoiding exposure to recognized risk factors, but also, as a complementary approach referred to as chemoprevention, by favouring the intake of protective factors. This study looked at the antiproliferative properties of two potential chemopreventive agents. Further studies are needed before

there could be specific recommendations on intake of individual isoflovanoids. Nevertheless, the ultimate goal is to utilize these phytochemicals in a primary prevention setting when it is addressed to healthy individuals, in order to inhibit the progression of the disease.

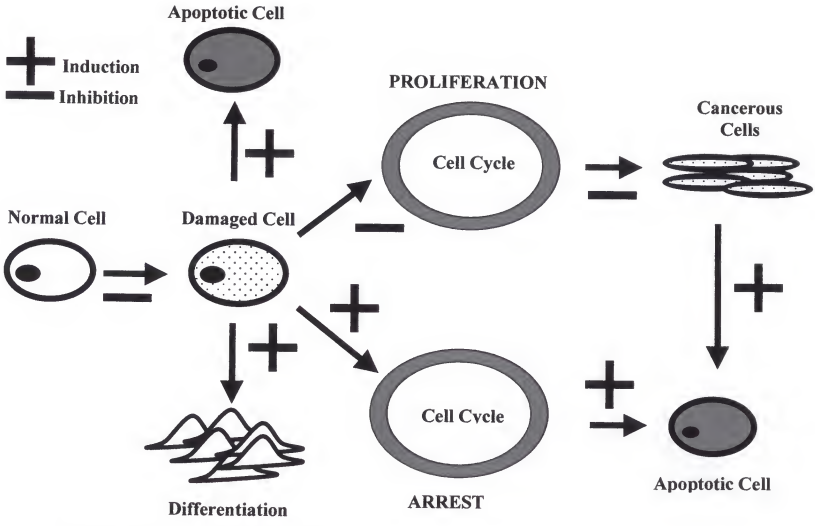


Figure 6-1. Mechanisms of chemoprevention.

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BIOGRAPHICAL SKETCH

Von G. Samedi was born October 3, 1972, in Port-au-Prince, Haiti. He received his elementary and secondary education at Saint-Louis de Gonzague, Port-au-Prince, Haiti, and graduated in the spring of 1991. The following fall, he moved to Boston, Massachusetts where he received his B.S. degree in chemistry from Suffolk University, in 1996. After graduation, he worked for a pharmaceutical company, AutoImmune Inc., Lexington, Massachusetts, until the fall of 1997 when he joined the graduate program in biomedical sciences at the University of Florida. He continued his doctoral studies under the mentorship of Dr. Kathleen Shiverick, in the Department of Pharmacology and Therapeutics. Besides his strong interest in biomedical research, he has always been interested in medicine. After completion of the Ph.D., he will start medical school in the fall of 2001, at the University of Florida, College of Medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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